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Ph.D. thesis

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Front page illustrations: Photo of a maize field from Shutterstock.com and a photo of emerging *Fusarium* spp. from pieces of maize.

PREFACE

Fungal contamination of food and feed is of growing concern for consumers and producers due to the possible risk of mycotoxin contaminaion. This is also true in Denmark where mycotoxin contaminated maize silage was suspected to be behind a series of health problems in dairy cattle herds. To secure a thorough investigation of this concern a large national collaboration between Technical University of Denmark (Department of Systems Biology and National Food Institute) Aarhus University (Faculty of Agricultural Sciences in Foulum and in Flakkebjerg), Danish Agricultural Advisory Service, the Danish Cattle Federation and the Danish Plant Directorate was launched. This thesis describes the determination of some of the most important fungi and their mycotoxins in Danish maize by developing novel isolation techniques and analytical chemical methods.

Most of the project was carried out at Center for Microbial Biotechnology at Department of Systems Biology, Technical University of Denmark with a three months stay at Faculty of Agricultural Sciences (Flakkebjerg), Aarhus University and a three months stay at Centraalbureau voor Schimmelcultures (CBS Fungal Biodiversity Center), Utrecht, the Netherlands. The project was financed by the Danish Directorate for Food, Fisheries and Agri Business Grant FFS05-3 and was supervised by Professor Ulf Thrane and Associate professor Birgitte Andersen.

Jens Laurids Sørensen

June 2009, Denmark

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Throughout my entire PhD project I have received fabulous guidance from my two supervisors Professor Ulf Thrane and Associate professor Birgitte Andersen for which I am forever thankful.

When I started on my PhD project I had only limited knowledge of analytical chemistry but patient and skillful guidance by Associate professor Kristian Fog Nielsen familiarized me with this area for which I am very thankful.

I am very grateful for the help and support I received from the staff at CBS Fungal Biodiversity Center, Utrecht, NL, during my three months stay. I would like to give a special thank to PhD student Maikel Aveskamp for introducing me to *Phoma* taxonomy and helping me with sequence analysis and to Professor Sybren de Hoog and research assistant Bert Gerrits van der Ende for their help and discussions on *Alternaria infectoria* sequence analysis. The stay was partly financed by "Christian og Ottilia Brorsons Rejselegat for yngre videnskabsmænd- og kvinder", "Otto Mønsteds Fond" and "Agronomfonden" for which I am thankful.

Professor Niels Bastian Kristensen (Aarhus University) is thanked for providing silage samples as are Associate professor Niels Henrik Spliid (Aarhus University) and Ghita Cordsen Nielsen for providing samples of fresh maize.

I thank Associate professors Ednar Wulff (Danish Seed Health Center) and Hans-Josef Schroers (Agricultural Institute of Slovenia) for inviting me to collaborate on their interesting research fields concerning the *Gibberella fujikuroi* species complex and wet apple core rot, respectively.

During my PhD project I have been very happy to have had several productive students under my wings. During a special *ad hoc* 10 ECTS project Jesper Mølgaard Mogensen laid the foundation for the development of an isolation medium for *Alternaria, Epicoccum* and *Phoma*. Dan Stenvall, Nikolaj Vynne and Tejs Kyhl later used this medium to isolate *Phoma* strains from maize in another special *ad hoc* 10 ECTS project. The contributions of these four students have been used in two papers and they have therefore been of great importance to my own PhD project.

I would also like to thank the technical staff, Anni, Kir, Hanne, Jesper and Lisette for helping me in the lab with instrumentation and fungal cultures.

My fellow PhD students at the center are also greatly appreciated for creating a wonderful working environment. Especially my office mate Ida is thanked for discussions and collaboration and for reading and commenting the thesis.

Finally I would like to thank my fiancée and my family and friends for encouragement and support as well as for their occasional mind-freeing distractions.

DANSK SAMMENDRAG

I løbet af de sidste ti år er brugen af majsensilage til fodring af malkekvæg nærmest steget eksponentielt. I den samme periode observerede landmænd en stigning i forekomsten i sundhedsmæssige problemer og mistanken faldt på at problemerne skyldes mykotoksiner i majsensilage, hvor specielt fusariumtoksiner blev fremsat som de hovedmistænkte. For at undersøge om disse problem i virkeligheden skyldtes mykotoksiner blev der startet et stort nationalt samarbejde mellem Danmarks Tekniske Universitet (Institut for systembiologi og Fødevareinstituttet), Aarhus Universitet (Det Jordbrugsvidenskabelige Fakultet i Foulum og Flakkebjerg), Landbrugets Rådgivningscenter, Dansk Kvæg og Plantedirektoratet.

Mykotoksinerne kan stamme enten fra de skimmelsvampe som inficerer majsplanter mens de gror på marken eller fra de som kan gro i ensilagestakkene. De mest velstuderede mykotoksinproducerende marksvampe er *Fusarium* og *Alternaria*, mens de vigtigste ensilagesvampe er *Penicillium roqueforti, Pen. paneum*, *Aspergillus fumigatus*, *Monascus ruber* and *Byssochlamys nivea*. Formålet med mit PhD projekt var at undersøge forekomsten af marksvampe samt nogle af deres vigtigste mykotoksiner i majs ved høst.

Da fusariumtoksiner var i det offentlige søgelys omhandlede de første undersøgelser de vigtigste af disse toksiner. Samarbejdspartnere fra Aarhus Universitet, Det Jordbrugsvidenskabelige Fakultet i Flakkebjerg og Landbrugets Rådgivningscenter bestemte forekomsten af bedst kendte fusariumtoksiner, heriblandt deoxynivalenol, nivalenol, T-2 and HT-2 toxin, zearalenone and fumonisin B1 og B2 i prøver indsamlet fra hele majsplanter ved høst over en fireårig periode (2004-2007). Deres resultater viste at selvom disse mykotoksiner forekom meget ofte i majsprøverne, så var niveauerne sjældent over de maximale grænseværdier fastsat i EU. Dette indikerer derfor at disse mykotoksiner ikke er den sandsynlige årsag til problemerne i malkekvægsbesætningerne.

I en undersøgelse of forekomsten af fusariumarter i majs observerede vi at den hyppigst forekomne art var *F. avenaceum*. Denne art producerer ikke nogle af de mykotoksiner som var inkluderet i den firårige undersøgelse, men derimod moniliformin og enniatiner. Jeg besluttede derfor at undersøge forekomsten af disse mycotoksiner. En metode til detektion af moniliformin blev udviklet ved hjælp af hydrophilic interaction chromatography (HILIC) med UV og MS detektion, hvilket var første gang at HILIC blev brugt til at analysere mykotoksiner. Denne metode blev brugt til at undersøge 28 prøver, hvori moniliformin blev fundet i de 15. Dog var niveauerne meget lave (<12 ppb). Selv om moniliformin betegnes som et potent mykotoksin er de observerede niveauer dog så lave at de højst sandsynlig ikke udgør en trussel for malkekvæg. En LC-MS/MS metode blev udviklet til simultan detektion af fire enniatiner (A, A1, B og B1) samt det relaterede stof beauvericin. Forekomsten af disse stoffer blev undersøgt i majsprøver indsamlet ved høst i to år (2005 og 2006). Resultaterne viste at enniatin B, som var den hyppigst forekomne analog, var til stede i 90 % af de undersøgte prøver i 2005 og 100 % i 2006 med maximale værdier på henholdsvis 489 ppb og 2598 ppb. Stabiliteten af enniatin B under ensilering blev undersøgt i ti ensilagestakke, hvor prøver udtaget efter 3, 7 og 11 måneder blev analyseret. Da enniatin B kunne detekteres i 11 måneder gammel ensilage tyder det på at stoffet er stabilt under disse forhold, selv om *F. avenaceum* ikke er i stand til at overleve i ensilagestakkene. Den lave forekomst af enniatinerne og beauvericin tyder dog på at disse stoffer heller ikke årsagen til problemerne. Ud fra disse observationer virker det derfor usandsynligt at fusariumtoksiner er involveret i problemerne i malkekvægsbesætningerne.

For at undersøge om andre marksvampe kunne være ansvarlige for problemerne besluttede jeg mig for at fokusere på de sort sporede svampe *Alternaria* og *Phoma*. Et semi-selektivt medium til isolering af disse to

familier blev udviklet og brugt til at undersøge deres forekomst i majsprøver indsamlet ved høst. *Alternaria infectoria* species-gruppe var det hyppigst forekomne *Alternaria* artskompleks efterfulgt af *A. tenuissima* og *A. arborescens*. Derimod blev der kun isoleret en *Phoma* art, *Ph. pomorum.* Et flersidet karakteriseringsstudie af *A. infectoria* species-gruppen viste at isolaterne fra majs ikke kunne differentieres fra isolater stammende fra andre habitater og regioner ved hjælp af morfologi, DNA sekvens analyse eller metabolit profiler. Isolaterne producerede infectopyroner, novae-zelandiner og altertoxin derivater på det kunstige vækstmedium dichloran Rose Bengal yeast extract sucrose agar (DRYES). På et kunstigt majsmedium producerede isolaterne dog kun små mængder af infectopyrones og novae-zelandins, hvorimod altertoxin derivaterne ikke kunne detekteres.

Metabolit analyser af *Ph. pomorum* viste at denne art er i stand til at producere en lang række af isocoumariner, med diaportinic acid som den vigtigste analog. Evnen til at producere isocoumariner kunne bruges til at differentiere denne art fra de andre arter i *Phoma* sektionen *Peyronellaea*. Isocoumariner blev også produceret på det kunstige majsmedium, dog i mindre mængder end på DRYES.

En LC-MS/MS metode blev udviklet til detektion af infectopyroner og diaportinic acid og blev, i et lille studium, brugt til at undersøge deres forekomst i 10 majsprøver. Hverken infectopyronerne eller diaportinic acid blev dog detekteret i nogle af de 10 prøver, hvilken kan indikere at disse stoffer ikke udgør en fare for malkekvæg som spiser majsensilage. Et mere udførligt studium er dog nødvendigt for at dette kan konkluderes endeligt.

Konklusionerne af undersøgelserne af forekomsten af mykotoksinerne produceret af *Fusarium*, *Alternaria* og *Phoma* indikerer at de ikke er årsagen til de problemer som landmænd har observeret i deres besætninger. Hvis problemerne skyldes mykotoksiner er de derfor højst sandsynligt ikke produceret i marken, men derimod i ensilagestakken. Fremtidig forskning skal derfor fokusere på udvikling af metoder til detektion af mykotoksiner produceret af ensilagesvampe.

SUMMARY

During the last ten years the use of maize silage in dairy cattle herds has increased almost exponentially. In the same period farmers experienced an increase in health problems in their herds, which was blamed on mycotoxins contamination of maize silage and especially *Fusarium* mycotoxins were put forward as the main suspects. To examine whether these problems in fact were caused by mycotoxins a large national collaboration between Technical University of Denmark (Department of Systems Biology and National Food Institute) Aarhus University (Faculty of Agricultural Sciences in Foulum and in Flakkebjerg), Danish Agricultural Advisory Service, the Danish Cattle Federation and the Danish Plant Directorate was launched.

The mycotoxins can be produced either by preharvest fungi infecting maize while it is growing in the fields or by fungi able to grow in the silage stacks. The best studied mycotoxin producing field fungi are *Fusarium* and *Alternaria*, whereas the most important fungi growing in the silage are *Penicillium roqueforti, Pen. paneum*, *Aspergillus fumigatus*, *Monascus ruber* and *Byssochlamys nivea*. The aim of my PhD. thesis was to examine the presence of preharvest fungi and some of the most important mycotoxins at harvest.

Fusarium mycotoxins were getting most public attention and therefore initial surveys were with some of the most important *Fusarium* mycotoxins. Project partners at Aarhus University, Faculty of Agricultural Sciences in Flakkebjerg and Agricultural Advisory Service determined the occurrence of the best studied *Fusarium* mycotoxins including deoxynivalenol, nivalenol, T-2 and HT-2, zearalenone and fumonisins B1 and B2 in samples of whole maize collected at harvest over a four year period (2004-2007). Their results showed that although most of these mycotoxins were very common contaminants of maize, the levels rarely exceeded the maximum levels set by the Commission for the European Communities. This indicates that these mycotoxins are not the likely cause of the health problems in dairy cattle herds.

In a screen of the presence of *Fusarium* in maize we observed that the most common species was *F. avenaceum*, which does not produce any of the mycotoxins included in the four year survey by our project partners. Instead, this species produces moniliformin and enniatins and I therefore decided to develop methods to determine their occurrence. A method for detection of moniliformin using hydrophilic interaction chromatography (HILIC) with UV and MS detection was developed, which was the first time HILIC was used for detection of mycotoxins. The method was used to survey 28 maize samples and the compound was detected in 15 samples, but only at trace levels (<12 ppb). Although moniliformin is considered a potent mycotoxin the observed levels were so low that this compound was also not considered as a threat to dairy cattle health. A LC-MS/MS method was developed for simultaneous detection of four enniatins A, A1, B and B1 and the related compound beauvericin. The occurrence of these compounds was surveyed in maize samples collected at harvest in 2005 and 2006. The result showed that enniatin B, which was the predominant analogue, was present in 90% of the samples in 2005 and 100% in 2006 with maximum levels of 489 ppb and 2598 ppb, respectively. The stability of enniatin B during the ensiling process was examined in 10 silage stack where samples of 3, 7 and 11 months old silage were collected and analyzed. Enniatin B could still be detected in 11 months old silage indicating that it is well conserved even though *F. avenaceum* does not survive the ensiling process. The low levels of enniatins and beauvericin indicated, however, that these compounds are not the cause of the problems. Based on these observations it appears therefore unlikely that *Fusarium* mycotoxins are involved in the problems observed at Danish dairy cattle farms.

To examine whether mycotoxins from other fungi, which can infect growing maize, could be responsible for the problems I then focused on the Dematiaceous fungi *Alternaria* and *Phoma*. A semi-selective medium was developed for isolation of these two genera and used to survey maize samples collected at harvest. *Alternaria infectoria* was the predominant *Alternaria* species-group followed by *A. tenuissima* and *A. arborescens*, whereas *Phoma pomorum* was the only *Phoma* species isolated from maize. A polyphasic study of the *A. infectoria* species-group showed that the strains isolated from maize were indistinguishable from strains from various sources by morphology, DNA sequence analysis and metabolite profiling. The strains produced infectopyrones, novae-zelandins and altertoxin derivates on the dichloran Rose Bengal yeast extract sucrose agar (DRYES). On an artificial maize medium, these strains only produced small amount of infectopyrones and novae-zelandins, whereas the altertoxin derivates were not detected.

Metabolite profiling of *P. pomorum* showed that it able to produce various isocoumarins with diaportinic acid as the predominant analogue. The ability to produce isocoumarins was a feature which was used to differentiate this species from other closely related *Phoma* species in polyphasic characterization of members of the *Phoma* section *Peyronellaea*. Isocoumarins were also produced by *Ph. pomorum* on the artificial maize medium, although in smaller amounts than on DRYES.

A LC-MS/MS method was developed for simultaneous detection of infectopyrones and diaportinic acid and used to examine ten maize samples in a small experiment. Neither infectopyrones nor diaportinic acid was detected in any of the ten samples, which may indicate that these compounds are not a threat to dairy cattle fed maize silage. A more thorough survey of these compounds is however still needed to fully exclude them.

The conclusions of the surveys of *Fusarium, Alternaria* and *Phoma* mycotoxins suggest that they are most likely not the cause of the problems observed in the dairy cattle farms. If the problems are mycotoxin related then they are not produced in the fields, but are derived from fungi able to grow in the silage stacks. Future research should therefore focus on the development of methods for detection of mycotoxins produced by these fungi.

ABBREVIATIONS

AcDON	Acetyl deoxynivalenol
BEA	Beauvericin
DAS	Diacetoxyscirpenol
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
ENNs	Enniatins (A, A1, B, B1)
FB	Fumonisin B
FUP	Fusaproliferin
FX	Fusarenone X
GPD	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High-performance liquid chromatography
HT-2	HT-2 toxin
ITS	Internal transcribed spacer
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MAS	Monoacetoxyscirpenol
MON	Moniliformin
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NIV	Nivalenol
PCR	Polymerase chain reaction
SCR	Scirpentriol
Spp.	Species
TEF-1α	Translation elongation factor 1α
T-2	T-2 toxin
UV	Ultraviolet
ZEA	Zearalenone

LIST OF ORIGINAL MANUSCRIPTS

The thesis includes eight original papers of which five are published, one is submitted and two is in preparation. In the text the papers are referred to by first author as well as their Roman numerals.

I. Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart Postharvest Review* **4:** 6.

II. Sørensen, J.L., Nielsen, K.F., and Thrane, U. (2007) Analysis of moniliformin in maize plants using hydrophilic interaction chromatography. *Journal of Agricultural and Food Chemistry* **55:** 9764-9768.

III. Sørensen, J.L., Nielsen, K.F., Rasmussen, P.H., and Thrane, U. (2008) Development of a LC-MS/MS method for analysis of enniatins and beauvericin in whole fresh and ensiled maize. *Journal of Agricultural and Food Chemistry* **56:** 10439-10443.

IV. Sørensen, J.L., Mogensen, J.M., Thrane, U., and Andersen, B. (2009) Potato carrot agar with manganese as an isolation medium for *Alternaria*, *Epicoccum* and *Phoma*. *International Journal of Food Microbiology* **130:** 22-26.

V. Andersen, B., Sørensen, J.L., Nielsen, K.F., van den Ende, A.H.G. and de Hoog, S. (2009) A polyphasic approach to the taxonomy of the *Alternaria infectoria* species-group. *Fungal Genetics and Biology* **46**: 642-656.

VI. Sørensen, J.L., Aveskamp, M.M., Thrane, U., and Andersen, B. (2009) Polyphasic characterization of *Phoma pomorum* isolated from Danish maize. (Submitted to *International Journal of Food Microbiology* 1st May 2009).

VII. Sørensen, J.L., Phipps, R.K., Nielsen, K.F., Frank, J., Schroers, H.J., and Thrane, U. (2009) Analysis of *Fusarium avenaceum* metabolites produced during wet apple core rot. *Journal of Agricultural and Food Chemistry* **57:** 1632-1639.

VIII. Wulff, E., Sørensen, J.L., Lübeck, M., Nielsen, K.F., Thrane, U., and Torp, J. (2009) Genetic diversity, extrolite production and pathogenicity of *Gibberella fujikuroi* species complex associated with rice seeds originating from Africa and Asia. *Environmental Microbiology and Environmental Microbiology Reports* (Accepted with minor revisions 30th June 2009)

CONTENTS

INTRODUCTION

PROJECT BACKGROUND

Maize (*Zea maydis* L.), also called corn in Australia, Canada and USA, is one of the most widely grown crops worldwide and constitutes the staple food in many regions. Globally, maize covered an area of 159 million hectares in 2007 yielding 794 million tons of kernels, which exceeded that of any other cereal (FAO, 2008). Besides being used for human consumption maize is extensively used in animal feed. This is also true in Denmark where the use of maize in cattle diet has increased massively during the last 10 years from 42 700 hectares (1.65 M. tons) in 1997 to 144 600 hectares (5.25 M. tons) in 2007 (Statistics Denmark, 2008) as seen in **Figure 1**. The increased use of maize is mainly due to the introduction of new early flowering varieties that are adjusted to the Danish climate, which has led to an almost complete replacement of the more laborious production of fodder beets.

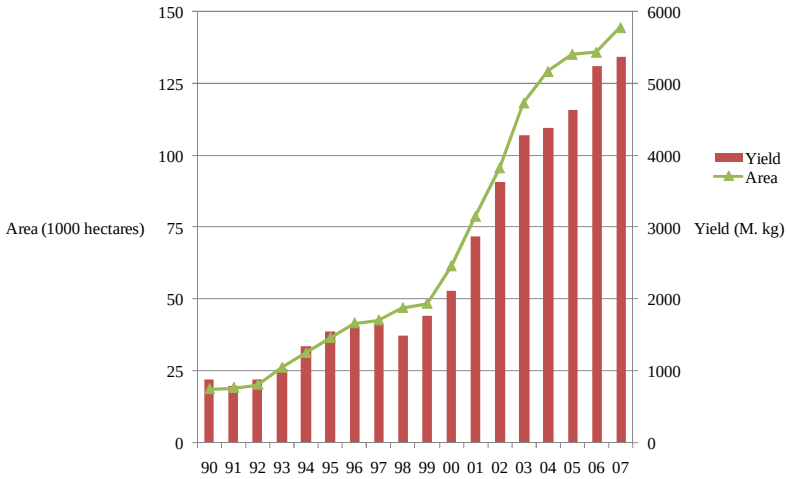


Figure 1. Area (1000 hectares) and yield (M. kg) of Danish maize 1990 – 2007 (Statistics Denmark, 2008).

Nearly all Danish maize is fed to cattle as maize silage, which is made from whole maize plants cut 15 – 20 cm above the ground into small pieces (5–10 mm × 10–30 mm). The maize pieces are then compiled in large stacks, compressed and sealed with plastic. Microbial respiration will rapidly create an environment with very low oxygen and high carbon dioxide levels. Anaerobic respiration by lactic acid bacteria is then responsible for lowering pH down to approximately 4. The low oxygen and acidic environment in the silage stack will ideally prevent contamination of undesired microorganisms, including filamentous fungi, and maize silage can therefore be stored and used for more than a year after harvest. Some fungal genera, such as the typical storage fungi *Aspergillus*, *Byssochlamys*, *Monascus* and *Penicillium* are however able to grow in silage stacks under the right circumstances, which can occur if the silage stack has not been properly sealed or if inadequate compression has led to formation of air tunnels (Storm et al., 2008) (**I**). Fungal contamination of the silage stacks can be a potential problem to the farmers due to reduced feed quality and production of toxic secondary metabolites known as mycotoxins. Mycotoxins can however also be

formed while the maize is growing in the field. The dominating toxigenic preharvest fungi include the genera *Aspergillus*, *Alternaria* and *Fusarium* (Storm et al., 2008) (**I**). The mycotoxins are very divers both structurally and in their toxic mode of action. Cattle feeding on mycotoxin contaminated maize silage can therefore be affected in a number of ways, although it is assumed that ruminants are more tolerant to mycotoxins than other animals due to the microorganisms in their digestive system, which are able to degrade or transform many harmful compounds (Fink-Gremmels, 2008).

In 2003 and 2004 much focus was put on mycotoxins in maize silage by agricultural newspapers in Denmark. Several stories linking mycotoxins, especially *Fusarium* mycotoxins, to health problems in dairy cattle herds were published (Houmann, 2003). Analysis of blood and milk samples from herds suspected to have been feeding on maize silage contaminated with *Fusarium* mycotoxins were performed, which showed extremely high levels of *Fusarium* mycotoxins (Houmann, 2003; Houmann, 2004; Mortensen, 2003; Woller, 2004). The link between mycotoxins and health problems was challenged by various veterinarian experts, who claimed that the health problems were not caused by mycotoxins but by well-known cattle diseases like Jejunal haemorrhagic syndrome or Johne´s disease (Jørgensen et al., 2004). Preliminary studies from the Danish Cattle Federation showed that levels of *Fusarium* mycotoxins in Danish maize were low and that they should not be regarded as a problem for Danish cattle (Møller and Thøgersen, 2003). Regardless of the data speculations continued and therefore a large national research collaboration titled "Mycotoxin carry-over from maize silage via cattle into dairy products" was launched in 2005. Project partners from Technical University of Denmark (Department of Systems Biology and National Food Institute) Aarhus University (Faculty of Agricultural Sciences in Foulum and in Flakkebjerg), Danish Agricultural Advisory Service, the Danish Cattle Federation and the Danish Plant Directorate joined forces to ensure an in depth investigation of this complex field. One of the major aims of the collaboration was to determine the mycobiota of fresh and ensiled maize and based on these results molecular tools for detection and quantification of important fungi in fresh and ensiled maize would be developed. Methods for detection of relevant mycotoxins would also be developed and the toxicity of some of these mycotoxins would be determined using various cell lines.

This thesis describes the isolation and identification of *Fusarium* and Dematiaceous fungi (*Alternaria*, *Epicoccum* and *Phoma*) from fresh maize sampled at harvest. These genera where examined because they are the predominant toxigenic preharvest fungi infecting maize in temperate regions. Maize grown in warmer areas, like the corn belt in Midwestern USA, can also be highly infected with *Aspergillus*, but this genera is generally thought to be absent in colder climates. A case of mycotoxicosis caused by *Diplodia maydis* toxins was reported in Argentina when 10 heifers died from eating moldy maize (Odriozola et al., 2005). *Diplodia maydis* has however not been observed in European maize and the species is therefore not considered to be a threat to cattle health in Denmark.

Based on the mycobiota surveys, methods for detection and quantification of relevant mycotoxins were developed and used to examine their occurrence in fresh and ensiled maize.

FUSARIUM: TAXONOMY, OCCURRENCE AND METABOLITE PROFILES

The anamorph genus *Fusarium* (teleomorph: *Gibberella, Albonectria* and *Haematonectria*) comprises more than 80 recognized species (Leslie and Summerell, 2006) of which several are destructive pathogens of cereals and maize occurring world wide. The genus has historically been segregated into large sections based on morphological and physiological characteristics, such as sporulation and coloration. One of the major milestones in *Fusarium* taxonomy is "Die Fusarien" (Wollenweber and Reinking, 1935), in which the genus was divided in 16 sections containing 65 species and 77 sub-specific varieties and forms. Prior to this publication more than a thousand *Fusarium* species existed, but with this milestone order was brought to *Fusarium* taxonomy. Many of the sections described by Wollenweber and Reinking (1935) are still commonly used and the platform for modern *Fusarium* is therefore derived from "Die Fusarien". Several changes to this system have been suggested after its introduction. The *Fusarium* genus was later simplified to contain nine species in the 1940s and 1950s, which was mainly achieved by introducing the *formae speciales* concept (Snyder and Hansen, 1945). The *formae speciales* were differentiated by the ability of their members to cause a wilt disease on a limited taxonomic range of host plants. The taxonomical system suggested by Snyder and Hansen was and is much debated, but it is still used for *F. oxysporum* and *F. solani*, although several phylogenetic analyses have challenged the *formae speciales* naming system in *F. oxysporum* complex (Baayen et al., 2000; O'Donnell et al., 1998b). The taxonomical system by Wollenweber and Reinking (1935) formed the basis of important revisions of the *Fusarium* genus (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson et al., 1981) and so the grouping of the genus into the sections remained. Although this segregation in many ways was a useful guide to *Fusarium* taxonomy, recent phylogenetic studies have however shown that the sections are polyphyletic and hence not longer applicable (Kristensen et al., 2005; Waalwijk et al., 1996). A new road map for navigation in the *Fusarium* genus is therefore needed. **Figure 2** shows a simple phylogenetic tree based on the translation elongation factor 1α (TEF-1α) using aligned sequences obtained from the *Fusarium* sequence database FUSARIUM ID-v. 1.0 (Geiser et al., 2004) with settings listed in **Appendix 1**.

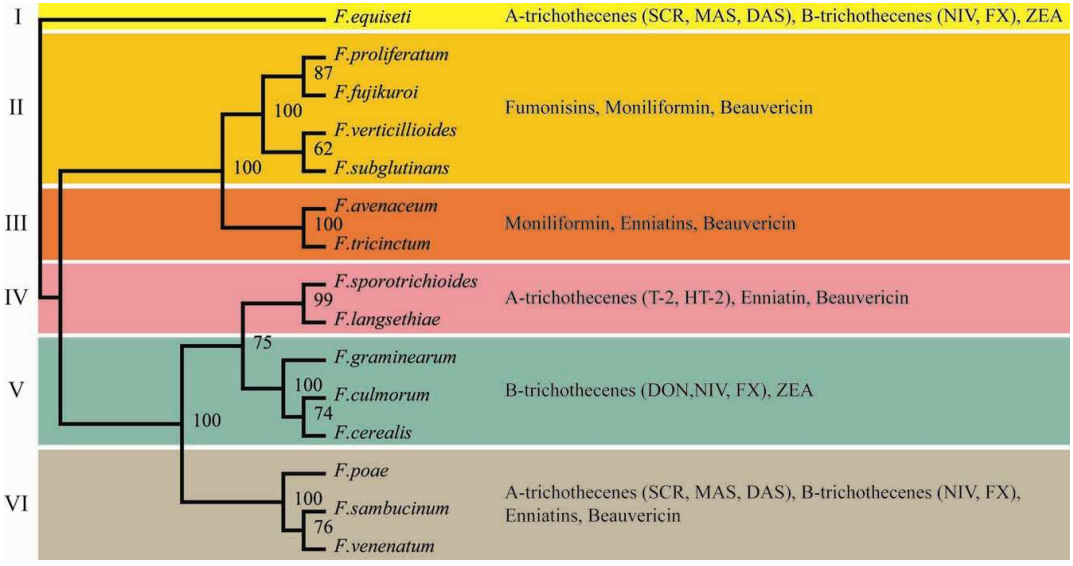


Figure 2. One most-parsimonious tree derived from TEF-1α sequences obtained from the FUSARIUM ID-v. 1.0 database rooted with *F. equiseti*. Important mycotoxins produced by members of the six clades are included. Bootstrap values >50% from 1000 replications are indicated at the internodes.

The tree consists of six clades, which concur with the morphology and metabolite profiles. *F. equiseti* is the sole member of clade I. This species is a cosmopolitan soil saprophyte, which is especially common in sub-tropical and tropical areas. It is also associated with dead or dying plant tissue and can cause damage to fruits. It is also associated with cereal grains in temperate regions including Northern Europe and Scandinavia (Andersen et al., 1996; Kosiak et al., 2003; Schumann et al., 1991b). Two morphological types of *F. equiseti* were isolated from Norwegian cereals (Kosiak et al., 2003). The predominant type had macroconidia with short apical cells, while the other rare type had long apical cells. It is however still unknown if the two morphologically different types also differ in other features, including metabolite production. The metabolite profile of *F. equiseti* is fairly different from the other *Fusarium* species as it is able to produce zearalenone (ZEA) in addition to the type A trichothecenes scirpentriol (SCR), monoacetoxyscirpenol (MAS) and diacetoxyscirpenol (DAS) and the type B trichothecene nivalenol (NIV) and its acetylated derivate 4-acetyl nivalenol (=Fusarenone X (FX)). This ability to produce ZEA and both types of trichothecenes is unique. In addition to these compounds *F. equiseti* can also produce fusarochromanone, equisetin, and chrysogine (Hestbjerg et al., 2002). F. equiseti was reported to produce T-2 toxin and butenolide (Burmeister et al., 1971), but this was not confirmed by Hestbjerg et al (2002).

Clade II contains *F. verticillioides* (=*F. moniliforme*; *G. moniliformis*), *F. proliferatum* (*G. intermedia*), *F. subglutinans* (*G. subglutinans*) and *F. fujikuroi* (*G. fujikuroi*), which are members of the *Gibberella fujikuroi* species complex. The *Gibberella fujikuroi* species contains at present ten recognized mating types/species (MP A-J), which were summarized in Leslie et al. (2007). *F. fujikuroi* (MP C) is the causal agent of bakanae disease of rice, whereas *F. verticillioides* (MP A), *F. proliferatum* (MP D) and *F. subglutinans* (MP E) are mainly associated with cereals and maize in subtropical and tropical regions. *F. verticillioides* and *F. proliferatum* are considered to be among the most important *Fusarium* species alongside *F. culmorum* and *F. graminearum* in cereals and maize due to their destructive and widespread nature. Members of this clade differ from the other clade in their ability to produce carcinogenic fumonisins, although strains of *F. fujikuroi* produce no or only small amounts of fumonisins. Unlike the three other species, moniliformin and fusaproliferin are not produced by *F. verticillioides* isolated from cereals and maize (Moretti et al., 2004; Schütt et al., 1998). *F. verticillioides* strains isolated from banana, which represent a separate genetic lineage than strains from cereals and maize, did however produce moniliformin (Moretti et al., 2004). In addition to these compounds, members of this clade can also produce fusarins, fusaric acid and beauvericin, although beauvericin is only produced in small amounts by *F. verticillioides*. *F. fujikuroi* is able to produce gibberellins as the only *Fusarium* species, although the other members of clade II also possess the required gene cluster (Malonek et al., 2005).

Clade III contains *F. avenaceum* and *F. tricinctum* which are among the most common *Fusarium* species in cereals in temperate area like northern Europe (Andersen et al., 1996; Bottalico and Perrone, 2002; Kosiak et al., 2003). *F. avenaceum* and *F. tricinctum* are able to produce a wide range of metabolites including moniliformin and enniatins (Langseth et al., 1999; Schütt et al., 1998; Uhlig et al., 2006a) and in rare cases beauvericin (Logrieco et al., 1998; Morrison et al., 2002).

Clade IV consist of *F. sporotrichioides* and *F. langsethiae* which are common contaminants of cereals in temperate to cold areas of the world. *F. langsethiae* was originally isolated as “powdery *F. poae*” as it has many morphological similarities to *F. poae* (Torp and Langseth, 1999). *F. langsethiae* is however closer related to *F. sporotrichioides* both chemotaxonomically (Thrane et al., 2004; Torp and Langseth, 1999) and phylogenetically (Knutsen et al., 2004; Konstantinova and Yli-Mattila, 2004; Wilson et al., 2004). These two

species are the main sources of the type A trichothecenes T-2 and HT-2 toxin, although some strains of *F. poae* also produce these highly toxic mycotoxins. In addition to T-2 and HT-2 several other type A thrichothecenes are also produced by *F. sporotrichioides* and *F. langsethiae* including SCR, DAS, MAS and neosolaniol. Both species also produce culmorin, butenolide, aurofusarin and traces of the cyclic depsipeptides beauvericin and enniatins. *F. langsethiae* is a good producer of chrysogine, whereas only 1 of 35 *F. sporotrichioides* strains produced this metabolite (Thrane et al., 2004).

Clade V contains *F. cerealis* (Synonym: *F. crookwellense*), *F. culmorum* and *F. graminearum*, which are very common pathogens of small grain cereals and maize in areas with temperate climate, such as Northern Europe (Andersen et al., 1996; Kosiak et al., 2003) and New Zealand (Hussein et al., 2003), but *F. graminearum* is also a very frequent pathogen in warmer regions like Southern Europe (Logrieco et al., 2003). A segregation of *F. graminearum* into first nine (O'Donnell et al., 2004) and later eleven new species (Starkey et al., 2007) has been suggested based on phylogenetic evidence. Opposing evidence based on morphology (Leslie and Summerell, 2006) cross-fertility tests (Bowden et al., 2005) and phylogenetic (Leslie et al., 2007) suggest that the new species should be considered one and the same species, *F. graminearum*. Members of clade V produce zearalenone (ZEA) and ZEA derivates (α- and β-zearalenol) in addition to the type B trichothecenes deoxynivalenol (DON), 3-acetyl DON (3-ADON), 15-acetyl DON (15-ADON), nivalenol (NIV) and fusarenon X (FX = 4-acetyl nivalenol) (Thrane, 2001). Furthermore they produce chrysogine, aurofusarin, fusarins, butenolide, rubrofusarin and 2-acetylquinazolinone. Three B-trichothecene chemotypes has been identified, c*hemotype I*: (DON and (3-ADON), *chemotype II* (DON + 15-acetyl DON) and *chemotype II* (NIV and FX) (Miller et al., 1991). These three chemotypes are represented in *F. graminearum*, whereas *F. culmorum* only contains chemotype I and II. *F. cerealis* is considered a non-producer of DON and only *chemotype III* is present in this species.

Clade VI consists of *F. poae, F. sambucinum* and *F. venenatum*. *F. poae* is a common contaminant of cereals in temperate regions and resembles morphologically members of clade IV, especially *F. langsethiae*. Like *F. equiseti*, *F. poae* produce both type A and B trichothecenes as it is able to produce SCR, DAS and MAS (and more rarely T-2 and HT-2 toxin) in addition to NIV and FX (Thrane et al., 2004). *F. sambucinum* and *F. venenatum* produce the type A trichothecene DAS, but they are not producers of type B thrichothecenes (Thrane and Hansen, 1995). *F. venenatum* Nirenberg was raised to species levels when it was segregated from *F. sambucinum* sensu lato (Nirenberg, 1995). The production strain used to make the mycoprotein product Quorn® was identified as *F. venenatum* and not *F. graminearum* as originally suggested (O'Donnell et al., 1998a). The Quorn® production strain produced however only trace amounts of type A trichothecenes on an artificial rice medium and they could not be detected in Quorn® products (O'Donnell et al., 1998a).

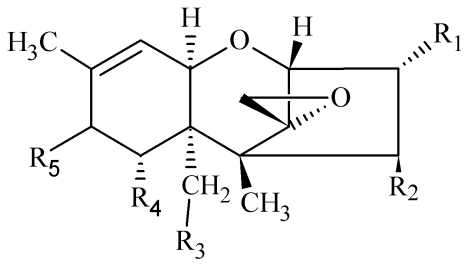
IMPORTANT FUSARIUM MYCOTOXINS: STRUCTURE, TOXICITY AND OCCURRENCE IN DANISH MAIZE

Although the Fusarium genus is a rich source of a wide variety of bioactive secondary metabolites, trichothecenes, zearalenones and fumonisins, have for many years been the major focus point in field surveys and toxicology studies. In recent years several other metabolites have emerged as possible important mycotoxins, which include moniliformin, enniatins, beauvericin and fusaproliferin. In this section the chemical structures and toxic modes of action of some of the most important *Fusarium* mycotoxins will

be described. Their occurrence in cereals and maize in Europe is described with a special focus on the Scandinavian situation.

TRICHOTHECENES

Trichothecenes are a group of structurally related compounds with a common tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system. The approximately 170 identified trichothecenes (Grove, 1988; Grove, 1993) can be divided in four types (A-D) according to variations in the functional hydroxyl and acetoxy side groups. Type A trichothecenes have an oxygen function at the C-8 position, whereas type B have a carbonyl function at this position (**Figure 3**). Type C trichothecenes have a second epoxide function, whereas D-type trichothecenes contain a macrocyclic ring between C-4 and C-15 with two ester linkages.



	R_1	R_2	R_3	R_4	R_5
SCR	OH	OH	OH	H	H
15-MAS	OH	OH	OAc	H	H
4, 15-DAS	OH	OAc	OAc	H	H
T-2	OH	OAc	OAc	H	OR_6[a]
HT-2	OH	OH	OAc	H	OR_6[a]
DON	OH	H	OH	OH	O
3-AcDON	OAc	H	OH	OH	O
15-AcDON	OH	H	OAc	OH	O
NIV	OH	OH	OH	OH	O
4-AcNIV	OH	OAc	OH	OH	O

Figure 3. Structures of important type A and B trichothecenes. [a] $R_6 = Me_2CHCH_2C(O)$

The simple trichothecene types A and B can be produced by several *Fusarium* species (Thrane, 2001), but also by some species of *Trichoderma* (Nielsen et al., 2005b), whereas macrocyclic trichothecenes can be produced by some species of *Myrothecium* and *Stachybotrys* (Grove, 1993).

The most important type A trichothecenes are T-2 toxin and scirpenol (SCR) of which T-2 toxin also occur in a de-acetylated form (HT-2 toxin), while SCR also occurs in mono-acetylated (MAS) and di-acetylated (DAS) forms. The most important type B trichothecenes are deoxynivalenol (DON; syn. vomitoxin) and nivalenol (NIV) of which DON also occurs in two mono-acetylated forms (3-AcDON and 15-AcDON), while NIV also occurs in mono-acetylated forms of which 4-AcNIV (Fusarenone-X) is the best studied.

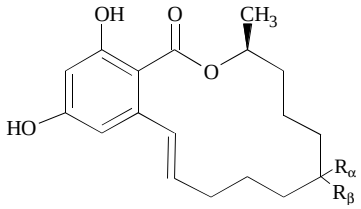
Studies have shown that the toxic mode of action of trichothecenes is due to inhibition of eukaryotic protein synthesis by binding to the 60S ribosomal subunit and by interaction with the enzyme peptidyltransferase (Cundliff et al., 1974; Cundliffe and Davies, 1977). This interaction leads to varying degrees of inhibition of peptide bond formation depending upon the chemical structure of the specific trichothecene (Cundliff et al., 1974; Cundliffe and Davies, 1977). The most severe effects of ingestion of trichothecene contaminated feed in life stock have been reported in swine and include reduced feed intake, feed refusal, skin irritation, vomiting, diarrhoea, and multiple haemorrhage (Chavez, 1984; Friend et al., 1982). Cattle on the other hand seem to be more tolerant to trichothecenes (Charmley et al., 1993; Noller et al., 1979; Trenholm et al., 1985) possibly due to the detoxifying potentials of the rumen microbes,

which, for instance, can metabolize DON to the less toxic de-epoxy-DON (DOM-1). The main producers of DON and NIV are *F. graminearum, F. culmorum* and *F. cerealis* and as they are very common contaminants of cereals and maize world wide, these mycotoxins are also ubiquitous. In a large survey of *Fusarium* mycotoxins in European cereals and maize grains DON was detected in 89 % of 520 analyzed maize grain samples in levels up to 8850 ppb, whereas NIV was detected in 34 % of the samples in levels up to 340 ppb (Gareis, 2003).

T-2 and HT-2 toxin are produced by the two members of clade IV, *F. sporotrichioides* and *F. langsethiae*, and by a few *F. poae* strains from clade VI (Thrane et al., 2004). SCR, MAS and DAS are also produced by these three species (Thrane et al., 2004), but also by some *F. equiseti* strains from clade I (Hestbjerg et al., 2002) and by *F. sambucinum* and *F. venenatum* from clade VI. As the producers of type A trichothecenes mainly are native to areas with temperate climate their mycotoxins are also found in these areas. T-2 and HT-2 toxin have been found in high levels in Norwegian cereals, where oats were the grain species most heavily contaminated with maximum detected levels of T-2 and HT-2 being 330 ppb and 880 ppb, respectively (Langseth and Rundberget, 1999). In the large European survey of *Fusarium* mycotoxins T-2 was found in 28 % of the analyzed maize grain samples, with the highest level (255 ppb) was found in Austria (Gareis, 2003). HT-2 was found in 24 % of the analyzed maize kernel samples and again the maximum levels were found in Austria (120 ppb).

ZEARALENONES

Zearalenone (ZEA, **Figure 4**) is an estrogenic metabolite mainly produced by *F. graminearum, F. culmorum, F. cerealis* and *F. equiseti*. These species can also produce small amounts of several related metabolites with α-zearalenol and β-zearalenol (α- and β-ZOL) being the most important derivates (Richardson et al., 1985). All zearalenones are estrogenic compounds although α-zearalenol has a higher estrogenic potential than ZEA and β-zearalenol (Hagler et al., 1979; Peters, 1972), probably due to a greater binding affinity to estrogen receptors (Fitzpatrick et al., 1989).



	R
Zearalenone	O
α-zearalenol	OH(α)
β-zearalenol	OH(β)

Figure 4. Chemical structure of zearalenone and α-zearalenol and β-zearalenol.

Binding of zearalenones to estrogen receptors result in a hyperestrogenicity syndrome in several animals (Zinedine et al., 2007). Ingested ZEA is biotransformed mainly in the liver to α- and β-ZOL in ratios varying between animal species (Malekinejad et al., 2006). This results in differentiated sensitivity to ZEA and pigs are more sensitive than other animals because they mainly transform ZEA to the more potent α-ZOL (Malekinejad et al., 2006). In the liver of cattle ZEA is mainly transformed to β-ZOL and the ratio of ZEA, α- and β-ZOL was (1:1:5) in a study where heifers were fed contaminated oats (Kleinova et al., 2002). The

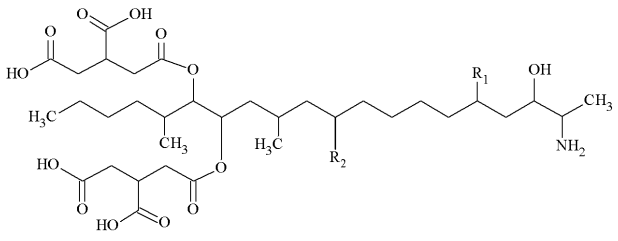
rumen microorganisms can also transform ZEA, although the ratio may be different as the ratio of ZEA, α- and β-ZOL was (30:40:30) in the duodenum of cows in a study by Dänicke et al. (2005).

In the large European screening for *Fusarium* mycotoxins ZEA was found in 32 % of approximately 5000 samples of cereal grains and cereal products tested (Gareis, 2003). ZEA was a frequent contaminant of maize grains occurring in 79 % of the analyzed samples. The occurrence of ZEA in cereals and maize in Scandinavia is however not well examined, although studies have shown that ZEA levels are low in Finnish cereals (Eskola et al., 2001; Hietaniemi and Kumpulainen, 1991).

FUMONISINS

Fumonisins are a group of mycotoxins with carcinogenic properties having a strong structural similarity to sphinganine, the backbone precursor of sphingolipids (Gelderblom et al., 1992). Sphingolipids are thought to play an important part in signal transmission and cell recognition, but the sphinganine biosynthesis can be inhibited by fumonisins due to their close structural similarity (Wang et al., 1991). The fumonisins contain a 18-carbon backbone with varying side-groups and have traditionally been classified into four series A, B, C and P (Bartok et al., 2006; Rheeder et al., 2002), although a new series, PHFB(4)OSA, has recently been identified (Bartok et al., 2008). The fumonisin B (FB) series, which includes the toxicologically important FB1, FB2 and FB3, (**Figure 5a**) are the most abundantly occurring fumonisins, with FB1 as the predominant analogue in this series. Fumonisins are primarily produced by some members of the *Gibberella fujikuroi* species complex with *F. proliferatum* and *F. verticillioides* as the chief producers (Leslie et al., 1992). These species are mainly present in tropical and subtropical areas and fumonisin contaminations of preharvest crops are therefore higher in these areas. FB1 was detected in 66 % of the 801 analyzed maize samples in levels up to 10.2 ppm in the large survey of *Fusarium* mycotoxins (Gareis, 2003). Interestingly, *Aspergillus niger* has recently been identified as a producer of two fumonisin analogues FB2 and FB4 (Frisvad et al., 2007). A fumonisin-like compound, 2-AOD-3-ol (**Figure 5b**), was isolated from *F. avenaceum*, but other species including *F. tricinctum, F. poae* and *F. langsethiae* may also be producers of this compound (Uhlig et al., 2005). The compound has however not been detected in naturally infected cereals yet, and it is also undetermined whether it possesses the same toxic potential as the fumonisins.

A



	R_1	R_2
FB1	OH	OH
FB2	OH	H
FB3	H	OH

B

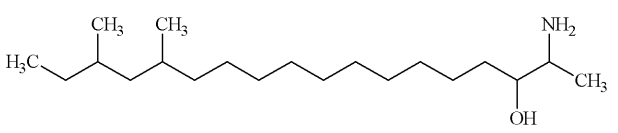


Figure 5. Chemical structures of FB1, B2 and B3 (A) and 2-AOD-3-ol (B).

OCCURRENCE OF TRICHOTHECENES, ZEARALENONE AND FUMONISINS IN DANISH MAIZE

In a survey over four years (2004-2007) the occurrence of DON, NIV, T-2, HT-2, ZEA, FB1 and FB2 was examined in whole fresh maize at harvest in a collaboration between Danish Agricultural Advisory Service, Danish Cattle Federation and Faculty of Agricultural Sciences at Aarhus University (Nielsen et al., 2005a; Nielsen et al., 2006; Nielsen et al., 2007; Nielsen et al., 2008). The results of this survey (summarized in **Table 1**) showed that the highest mean levels for all compounds, except FB2, occurred in 2006. While DON, NIV and ZEA were frequent contaminants, type A trichothecenes and fumonisins were more rarely observed in all years.

Table 1. Occurrence (ppb) of *Fusarium* mycotoxins in whole Danish maize at harvest 2004 - 2007. Data from (Nielsen et al., 2005a; Nielsen et al., 2006; Nielsen et al., 2007; Nielsen et al., 2008).

	2004 (n=67)			2005 (n=66)			2006 (n=74)			2007 (n=32)		
	(%)[a]	Mean[b]	Max[c]	(%)	Mean	Max	(%)	Mean	Max	(%)	Mean	Max
DON	96	550	5440	97	670	3312	97	909	10780	94	609	2344
NIV	97	271	1528	82	239	1898	100	621	2956	88	231	1962
ZEA	34	18	308	33	19	221	62	131	2668	69	71	342
T-2	0	0	0	3	6	245	5	10	427	0	0	0
HT-2	1	1	41	6	7	166	15	27	910	0	0	0
FB1	1	1	56	0	0	0	11	45	2270	0	0	0
FB2	27	26	207	14	5	43	7	20	1155	0	0	0

[a] Positive samples (%). [b] Mean (ppb) calculated by setting levels below limit of quantification to 0. [c] Maximum level (ppb) detected.

The commission of the European communities has set the following maximum levels for maize to be used to feed cattles DON (8000 ppb), ZEA (2000 ppb) and FB1+FB2 (60.000 ppb) (Commission of the European Communities, 2006). There has not been set maximum levels for NIV, because the presence of NIV is normally highly correlated to DON, which has been accounted for when the tolerated DON values was established. The maximum levels for T-2 and HT-2 has not yet been established due to limited toxicological data and field surveys. A maximum level for the combined sum of T-2 and HT-2 is however expected to be established in 2009, and some have predicted that the maximum level is set to 500 ppb (Pettersson et al., 2008). During the four year survey only one sample, from 2006, exceeded the maximum levels for DON set by the commission of the European communities. The same sample was also the only one to exceed the maximum levels for ZEA, whereas the sum of FB1+FB2 was well below maximum levels in all samples. T2+HT-2 levels exceed 500 ppb in two samples, both in 2006, with the sum of the two compounds being 652 and 1336 ppb. Because the levels of the examined mycotoxins only rarely exceeded the established maximum levels through a four year period it can be concluded that the examined compounds can not be regarded as a threat to cattle health in Denmark. They are therefore unlikely to be the cause of health problems in dairy cattles in Denmark. The *Fusarium* genus is however a rich source of other bioactive secondary metabolites, which may be produced in high levels in Danish maize. A possible important source is *F. avenaceum*, which is one of the predominant species in cereals in Scandinavia and Northern Europe. This species is a producer of several secondary metabolites, which will be discussed in the following section.

FUSARIUM AVENACEUM METABOLITES

As mentioned previously *F. avenaceum* is one of the predominant *Fusarium* species in cereals in Scandinavia (Andersen et al., 1996; Jestoi et al., 2004; Kosiak et al., 2003). Danish maize is therefore likely to be contaminated with mycotoxins produced by this species. The metabolite profile of *F. avenaceum* includes an array of structurally very different bioactive compounds, ranging from small polar compounds to large apolar compounds, with moniliformin and enniatins being the best studied. Contamination of food and feed with moniliformin and enniatins are considered as an emerging toxicological problem (Jestoi, 2005).

MONILIFORMIN

Moniliformin (3-hydroxycyclobut-3-ene-1,2-dione, **Figure 6**) was first isolated as the sodium (Cole et al., 1973) and potassium salt (Springer et al., 1974) from *F. verticillioides* strains, which later were reidentified as *F. subglutinans*. The compound is a very strong acid with pKa estimated to be 0 - 1.7 (Bellus et al., 1978; Scharf et al., 1978; Steyn et al., 1978). It is produced by several *Fusarium* species in addition to *F. avenaceum*, with *F. tricinctum, F. proliferatum* and *F. subglutinans* as the most important sources (Schütt et al., 1998). Interestingly, *F. verticillioides* strains isolated from banana can also produce moniliformin, whereas strains isolated from maize are non-producers (Moretti et al., 2004).

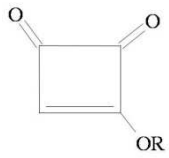


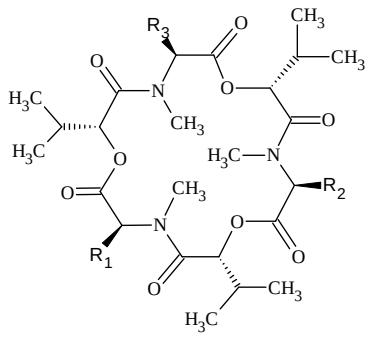
Figure 6. Structure of moniliformin. R = H, Na or K

It is an inhibitor of several thiamine pyrophosphate depending enzymes with pyruvate dehydrogenase as the best studied resulting in inhibition of gluconeogenesis (Burka et al., 1982; Pirrung et al., 1996). In addition, moniliformin has been reported to inhibit glutathione peroxidase and reductase (Chen et al., 1990). Orally fed synthetic moniliformin caused severe mycocardial lesions in cockerels, ducklings, rats and mice (Thiel, 1978) and it has been suggested to be connected to Keshan disease, which occurs in parts of China with high levels of moniliformin levels in maize, because affected persons had similar symptoms as test animals (Chen et al., 1990; Zhang and Li , 1989). Other surveys failed however to link moniliformin levels to the disease (Yu SR et al., 1995) and it is therefore still unknown if moniliformin is the cause of the disease. Moniliformin is not cytotoxic (Morrison et al., 2002; Uhlig et al., 2006a), but its oral toxicity is comparable to some of the strongest *Fusarium* derived mycotoxins such as type A trichothecenes (Kriek et al., 1977). The European Commission has not established maximum levels for moniliformin yet, but because it is supposed to be equally toxic as T-2 and HT-2 maximum tolerated levels could be in the area of 500 ppb. In Scandinavia, the presence of moniliformin has been examined in Finland and Norway. In the Finish survey of cereal grain samples (barley, oat, rye and wheat) moniliformin levels exceeded 500 ppb in 3 of 38 examined samples with the highest detected level being at 810 ppb (Jestoi et al., 2004). Similar levels were detected in Norwegian cereal grain samples where moniliformin was found in levels up to 950 ppb

with 3 of 231 samples surpassing 500 ppb. Worldwide analysis of maize and grain samples have detected moniliformin levels up to 2 mg/kg in Austrian cereal grain (Filek and Lindner, 1996) and 3.2 mg/kg in Gambian and South African maize and maize products (Sharman et al., 1991). These high moniliformin levels are likely to be produced by additional species than *F. avenaceum*, including *F. subglutinans* and *F. proliferatum* as *F. avenaceum* is not common in warm climate zones.

ENNIATINS AND BEAUVERICIN

Enniatins and beauvericin are a group of structurally related cyclic hexadepsipeptides consisting of three *d*-2 hydroxycarboxylic acid and -*N*-methylamino acid residues linked alternately (**Figure 7**). *F. avenaceum* can produce at least six enniatins and usually they occur in the following order: B > B1 > A1 > A > B2 > B3 (Uhlig et al., 2006a). In addition to enniatins some strains of *F. avenaceum* can also produce small amounts of beauvericin (Logrieco et al., 1998; Morrison et al., 2002). Enniatins can also be produced by several other species including *F. tricinctum, F. poae, F. sporotrichioides* and *F. langsethiae* (Thrane, 2001; Thrane et al., 2004), whereas beauvericin producers also include *F. sambucinum, F. poae, F. sporotrichioides* and *F. langsethiae*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (Logrieco et al., 1998; Thrane et al., 2004).



Enniatin A	R_1=R_2=R_3= -$CH(CH_3)CH_2CH_3$
Enniatin A1	R_1=R_2= -$CH(CH_3)CH_2CH_3$, R_3= -$CH(CH_3)_2$
Enniatin B	R_1=R_2=R_3= -$CH(CH_3)_2$
Enniatin B1	R_1=R_2= -$CH(CH_3)_2$, R_3= -$CH(CH_3)CH_2CH_3$
Beauvericin	R_1=R_2=R_3= -$CH_2C_6H_5$

Figure 7. Structures of beauvericin and enniatin A, A1, B and B1.

Enniatins and beauvericin are cytotoxic compounds (Uhlig et al., 2006a) and they have insecticidal (Grove and Pople, 1980), antibiotic (Carr et al., 1985; Ovchinnikov et al., 1974) and antifungal (Burmeister et al., 1977) properties. The apolar nature of enniatins and beauvericin enables them to be incorporated into cellular membranes in which they create cation selective channels (Ovchinnikov et al., 1974) and thereby disturb the intracellular ionic homeostasis (Kamyar et al., 2004; Kouri et al., 2003). The European Commission has not established guidelines for maximum levels of beauvericin and enniatins, mainly due to insufficient toxicological data. Beauvericin has been detected in maize kernels around the world (Bottalico et al., 1995; Chelkowski et al., 2007; Jurjevic et al., 2002; Ritieni et al., 1997; Srobarova et al., 2002) with levels ranging from trace amounts up to 520 ppm (Ritieni et al., 1997). The occurrence of enniatins in maize has not been examined as extensively as beauvericin, but Scandinavian surveys of cereal samples detected enniatin B in levels up to 18.3 ppm in Finland (Jestoi et al., 2004) and 5.8 ppm in Norway (Uhlig et al., 2006b). In these two surveys beauvericin occurred less frequently than the enniatins and the maximum level was 19 ppb in Finland (Jestoi et al., 2004) and 120 ppb in Norway (Uhlig et al., 2006b). These surveys

show that in other Scandinavian countries, where *F. avenaceum* is one of the dominating *Fusarium* species, there is also a possibility of high levels of enniatins in cereals, which may also be the case in Danish maize.

OTHER FUSARIUM AVENACEUM METABOLITES

Beside the already described metabolites several other metabolites can be produced by *F. avenaceum* (**Figure 8**). Of these compounds, fusarins, clamydosporols, chrysogine and butenolide are not well examined whereas more knowledge on aurofusarin, antibiotic Y and 2-Amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol) exists.

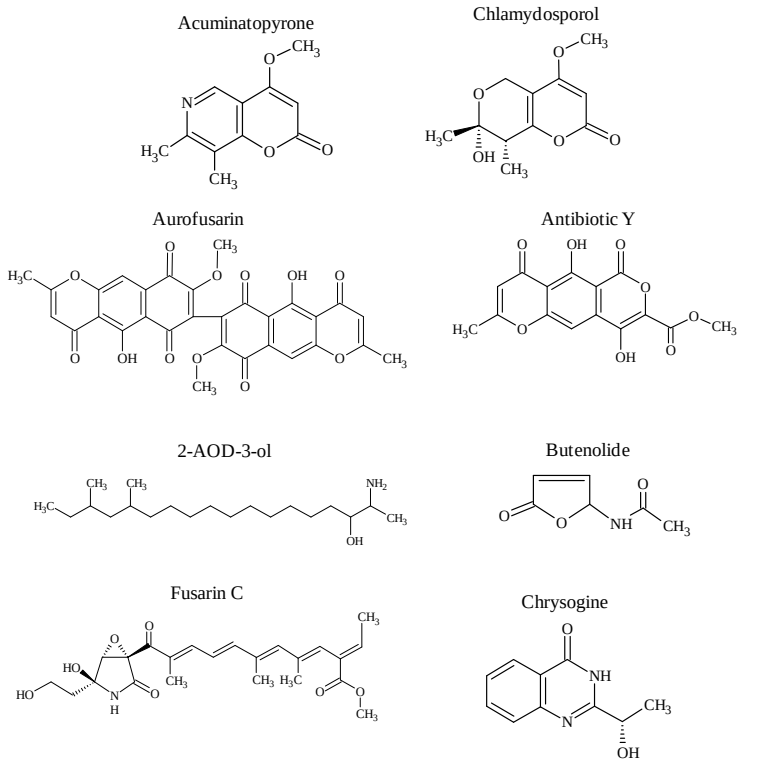


Figure 8. Structures of Fusarium avenaceum metabolites.

Aurofusarin is a red pigment of the naphthoquinone group produced by several *Fusarium* species including all members of clades III, IV, V and VI (**Figure 2**) (Thrane, 2001). Not much is known about the toxic effects of aurofusarin but it has been shown to have a negative effect on the antioxidant system of the quail egg yolk and to cause alterations in fatty acid composition of the egg yolk (Dvorska et al., 2001). Aurofusarin may also contain some cytotoxic properties as it was significantly related to cytotoxicity in two out of five assays tested by Uhlig et al. (2006a). In the only survey of aurofusarin in naturally infected samples it was found in wheat in levels up till 4.2 ppm (Kotik and Trufanova, 1998).

Antibiotic Y is a compound produced by several species including *F. avenaceum*, *F. lateritium*, *F. torulosum, F. acuminatum*, *F. flocciferum*, and *F. tricinctum* (Bushnell et al., 1984; Golinski et al., 1986; Samson et al., 2004; Thrane, 2001). The compound was originally isolated from *F. lateritium* as lateropyrone (Bushnell et al., 1984). The structure was later amended and the compound renamed antibiotic Y (Gorst-Allman et al.,

1986). As the name suggests it has antibiotic properties (Bushnell et al., 1984), but it could not be correlated to cytotoxicity (Uhlig et al., 2006a). The actual mechanism behind the antibiotic effects of the compound is, however, still unknown.

2-AOD-3-ol is a sphingosine analogue recently isolated from *F. avenaceum* (Uhlig et al., 2005). The compound is structurally similar to fumonisins and it is therefore speculated that it may have similar toxic properties. 2-AOD-3-ol has together with the enniatins been identified as the main cytotoxic factors produced by *F. avenaceum* (Uhlig et al., 2006a). The compound has not be detected in naturally infected samples yet, but 2-AOD-3-ol was produced in artificially inoculated wheat kernels (Uhlig et al., 2005). This suggests that *F. avenaceum* has the ability to produce the compound under field conditions and that 2-AOD-3-ol may also be present in Danish maize.

DEMATIACEOUS FUNGI RELEVANT TO DANISH MAIZE

Although *Fusarium* and their derived mycotoxins were accused of being the cause of health problem in dairy cattle herds, several other mycotoxin producing genera are able to infect maize. One of these genera is *Alternaria,* which is a common contaminant of cereals world wide including Denmark (Andersen et al., 1996). Other common genera found on cereals and maize are *Cladosporium*, *Epicoccum* and *Phoma* (Lauren and Di Menna, 1999; Macek and Zupan, 1993; Schumann et al., 1991a).

ALTERNARIA AND THEIR METABOLITES

After a recent revision the *Alternaria* genus contains now nearly 300 accepted species, which can be divided in two sections of small – or large spored species (Simmons, 2007). Small spored *Alternaria* are frequent contaminants of cereals and maize, while large spored species are rarely observed. The predominant small spored *Alternaria* infecting cereals belong to the *A. alternata, A. arborescens, A. tenuissima* and *A. infectoria* species groups (Andersen et al., 1996; Gonzalez et al., 1995; Kosiak et al., 2004; Müller, 1991; Schumann et al., 1991a). *A. alternata, A. arborescens* and *A. tenuissima* are closely related species, which cannot be differentiated by sequence analysis of some of the most commonly used phylogenetic markers like the Internal transcribed spacer region (ITS), glyceraldehyde-3-phosphate dehydrogenase (GPD), translation elongation factor 1α (TEF-1α) and β-tubulin (Peever et al., 2004; Pryor and Bigelow, 2003; Pryor and Gilbertson, 2000). The can however be separated morphologically (Simmons and Roberts, 1993) although *A. arborescens* may have been misidentified as *A. alternata* in some studies due to similar sporulation patterns (Simmons, 1999). The three species can also be separated with Random Amplification of Polymorphic DNA (RAPD) (Roberts et al., 2000) and with image analysis of pure cultures (Andersen et al., 2005). They can also be separated by metabolite profiling although they also have several metabolites in common including altenuene, alternariols, and altertoxins (**Figure 9**) (Andersen et al., 2002). Alternariols have been suggested to be associated with human esophageal cancer in an area of China with high consumption of *Alternaria* infected food (Liu et al., 1992). The carcinogenic effects of alternariol may be caused by their mutagenic properties (Brugger et al., 2006; Schrader et al., 2001) as they have been shown to induce DNA breakage and influence DNA synthesis and repair in *E. coli* (Xu et al., 1996) and mammalian hepatocytes (Liu et al., 1992). Altenuene is structurally similar to the alternariols (Pero et al., 1971), but as the toxicological data of the compound is sparse it is unknown if it also has similar

carcinogenic properties. Altenuene and alternariols have, however, been reported to be non-toxic in a chicken embryo cell assay (Griffin and Chu, 1983). Altertoxins (Altertoxin I, II and III) have, like alternariols, mutagenic properties (Schrader et al., 2001; Stack et al., 1986; Stack and Prival, 1986), but they have not been associated with cancer yet.

One of the most important groups of metabolites produced by *Alternaria* is tenuazonic acid (**Figure 9**) and derivates of this compound. *A. alternata* has been reported as a producer of tenuazonic acid (Shephard et al., 1991), however all three *A. alternata* strains examined by Andersen et al. (2002) did not produce this compound, whereas most *A. arborescens* and *A. tenuissima* strains did. Tenuazonic acid was first isolated from culture filtrates of a strain identified as *Alternaria tenuis* (syn *A. alternata*) (Rosett et al., 1957). The compound has been characterized as an anti-tumor agent (Gitterman et al., 1964; Shigeura and Gordon, 1963), but it also has antiviral (Miller et al., 1963), antibiotic (Gallardo et al., 2004; Gitterman, 1965), insecticidal (Cole and Rolinson, 1972) and cytotoxic (Zhou and Qiang, 2008) properties. The toxicity of tenuazonic acid may be the result of inhibition of protein synthesis, which has been reported in animal and plant cell cultures (Shigeura and Gordon, 1963; Umetsu et al., 1974; Zhou and Qiang, 2008).

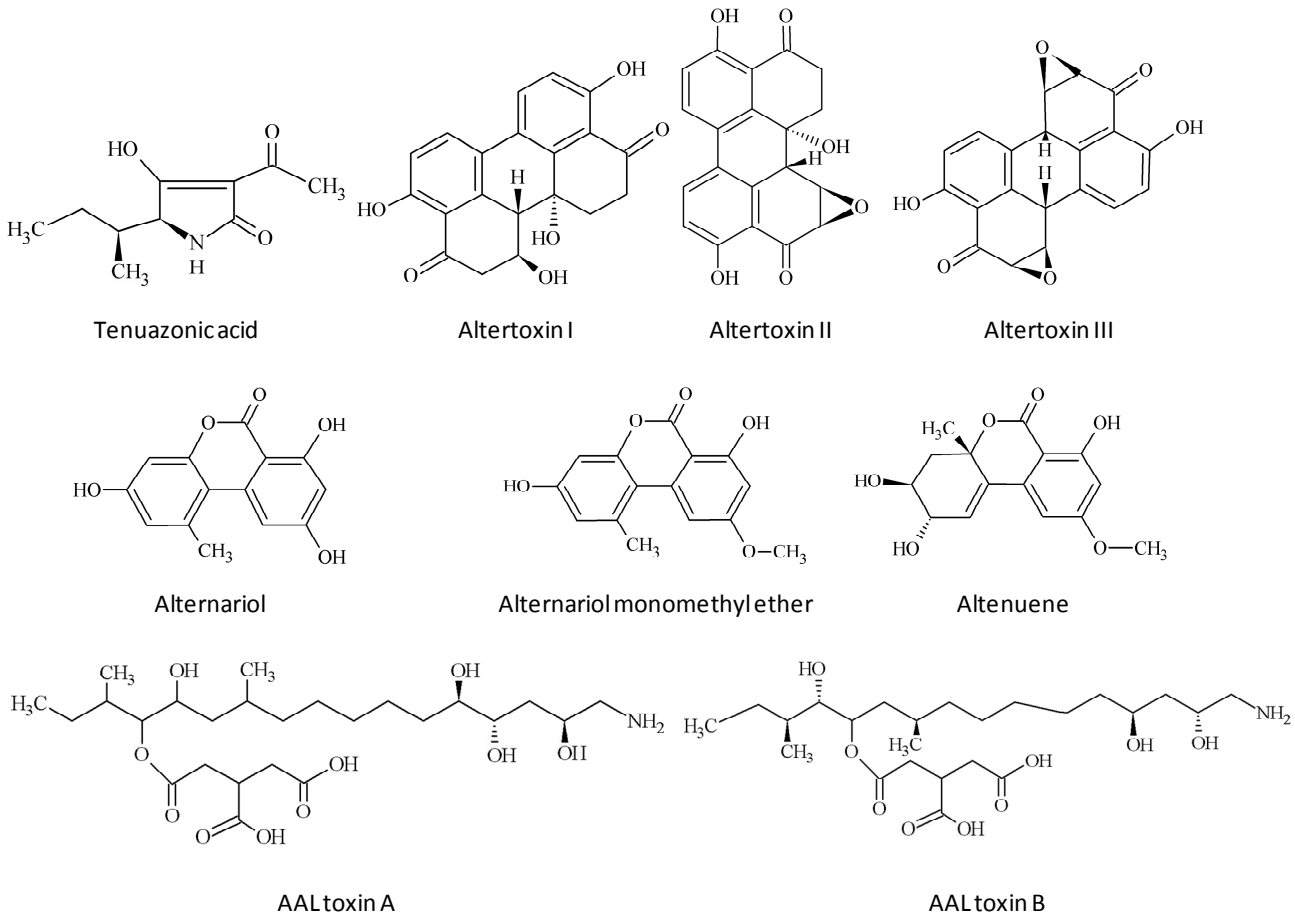


Figure 9. Important metabolites produced by *Alternaria alternata, A. arborescens* and *A. tenuissima*.

The natural occurrence of the above described *Alternaria* derived metabolites has been examined in maize or cereal samples on only a few occasions. The levels of alternariol, alternariol monomethyl ether and tenuazonic acid were examined in a two year survey of Argentinean wheat (Azcarate et al., 2008). In this survey alternariol was detected in 6% (maximum 1.4 ppm) of the 64 examined samples, whereas alternariol monomethyl ether and tenuazonic acid were detected in 23% (max. 7.5 ppm) and 19% (max. 8.8 ppm), respectively. Alternariol, alternariol monomethyl ether and tenuazonic acid were detected in 20 (max. 0.7 ppm), 21 (max. 1.4 ppm) and 22 (max 6.4 ppm), respectively, of 22 weathered wheat samples from China (Li and Yoshizawa, 2000). Altertoxin I could however not be detected from any of these samples. A different distribution of alternariol, alternariol monomethyl ether and tenuazonic acid was observed in weather damaged Australian wheat where the maximum levels detected was 1.1 ppm, 0.05 ppm and 0.2 ppm, respectively (Webley et al., 1997). The metabolites were however not detected in wheat grains without identifiable weather damage symptoms in the study by Webley et al. (1997). These studies show that these *Alternaria* metabolites may be present in high levels under the right circumstances.

The two sphingosine analogues AAL toxin A and B have been isolated from *A. alternata* f. sp. *lycopersici* (Bottini et al., 1981; Bottini and Gilchrist, 1981), which is a synonym for *A. arborescens* (Simmons, 1999). The two AAL toxins are similar to the very important *Fusarium* mycotoxins fumonisins and it can therefore be hypothesized that they possess similar carcinogenic properties. The AAL toxins have been included in a survey of sphingosine analogues in 30 and 40 maize silage stacks in 2001 and 2002 (Mansfield et al., 2007). In the survey by Mansfield et al. (2007) AAL toxin A was detected in levels ranging up till 2.0 ppm and AAL toxin B up till 0.9 ppm. The significance of these findings is somewhat unclear as not much is known about the toxicity of the AAL toxins. Cytotoxic experiments with several cell lines have shown that the cytotoxicity of AAL toxins is approximately 1-7 times lower than fumonisins B1 and B2 (Abbas et al., 1995; Shier et al., 1991). As the maximum tolerated levels of fumonisins in maize intended for animal feed is set to 60 ppm by the European commission (Commission of the European Communities, 2006), it is likely that the levels found by Mansfield et al. (2007) are nontoxic to the cattle feeding on the investigated maize silage.

The *A. infectoria* species-group includes presently the following morphologically recognized species: *A. arbusti* (Simmons, 1993), *A. avenicola* (Kwasna and Kosiak, 2003), *A. calycipyricola* (Roberts, 2007), *A. ethzedia* (Simmons, 1986), *A. hordeicola* (Kwasna et al., 2006), *A. infectoria* (Simmons, 1986), *A. intercepta* (Simmons and Roberts, 1993), *A. metachromatica* (Simmons, 1994), *A. novae-zelandiae* (Simmons and Roberts, 1993), *A. oregonensis* (Simmons, 1994), *A. triticimaculans* (Simmons, 1994), *A. triticina* (Prasada and Prabhu, 1962), *A. ventricosa* (Roberts, 2007) and *A. viburni* (Simmons and Roberts, 1993). When strains of this species-group are isolated from environmental samples they are often referred to as *A. infectoria* and many of these species have only been isolated once. The only species, in addition to *A. infectoria*, which continuously is being isolated, is *A. triticina*. This species was originally isolated from wheat in India (Prasada and Prabhu, 1962), but it has also been isolated from wheat in Argentina (Perello and Sisterna, 2006). Members of the *A. infectoria* species group differ in many ways from the other small spored *Alternaria* species. Unlike other groups of *Alternaria*, the most members of the *A. infectoria* group have a known sexual stage, *Lewia*. The species group can be morphologically separated from the other small-spored *Alternaria* species based on their sporulation pattern (Simmons and Roberts, 1993). Furthermore the *A. infectoria* species group form white colonies on Dichloran Rose bengal yeast extract sucrose agar (DRYES), whereas other small-spored *Alternaria* appear light green to dark green (Andersen et al., 2002).

Members of the *A. infectoria* species group have metabolite profiles that are significantly different from other small spored *Alternaria* species (Andersen et al., 2002). Chemical markers which can be used to differentiate the species group from other small spored *Alternaria* include infectopyrone, 4Z-infectopyrone and novae-zelandin A and B (**Figure 10**) (Christensen et al., 2005). Infectopyrone, but not the three other metabolites, is also produced by some species of the related genus *Ulocladium* and *Stemphylium* (Andersen and Hollensted, 2008; Christensen et al., 2005; Larsen et al., 2003). Infectopyrone was found not to be cytotoxic (Larsen et al., 2003), but it can be regarded as a potential mycotoxin due to the toxicity reported from structurally similar compounds (Ichihara et al., 1985; Kono et al., 1985). Novae-zelandin A and B are structurally related to the phytotoxic pyrenocines, produced by some large spored *Alternaria* (Montemurro and Visconti, 1992), but also by *Penicillium citrionigrum* (= *P. citreo-viride*) (Niwa et al., 1980) and *Pyrenochaeta terrestris* (Sato et al., 1979; Sparace et al., 1984). Whether novae-zelandin A and B also have phytotoxic properties is still unknown, but they do not possess antitumor or antiviral activity (Christensen et al., 2005).

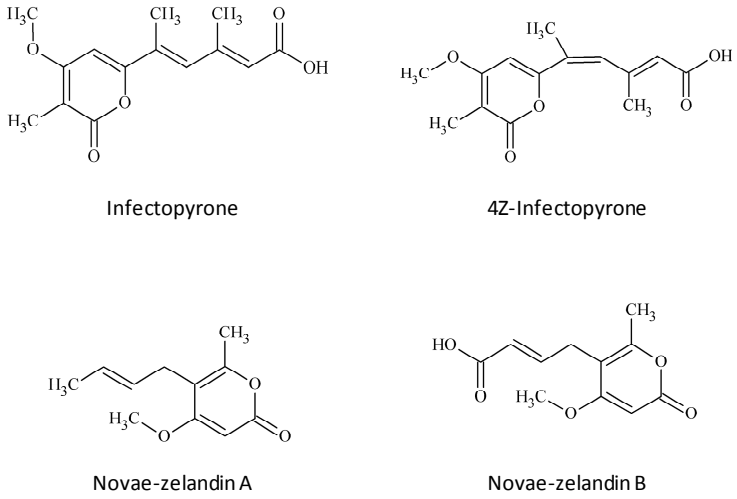


Figure 10. Metabolites produced by *Alternaria infectoria* species group.

The natural occurrence of metabolites produced by the *A. infectoria* species group has so far not been examined, although infectopyrone has been detected in moldy tomatoes infected with *Stemphylium eturmiunum* (Andersen and Frisvad, 2004). As members of the *A. infectoria* species group are common contaminants of cereals and maize it is likely that these commodities contain infectopyrones or novae-zelandins.

OTHER IMPORTANT DEMATIACEOUS FUNGI

Several additional producers of bioactive secondary metabolites are often associated with cereal and maize including species of *Epicoccum, Cladosporium, Stenocarpella* and *Phoma*. The natural occurrence of mycotoxins produced by these genera in food and feed has not been studied yet and an estimation of their importance is therefore not possible.

Mycotoxins produced by *Stenocarpella maydis* (teleomorph: *Diplodia maydis*) has been suggested as the primary cause in an Argentinean case where 10 heifers died from eating moldy maize infected with this species (Odriozola et al., 2005). *S. maydis* can produce diplodiatoxin, which contains a β-ketol side chain and the rare β,γ-unsaturated acid unit (Steyn et al., 1972). The compound induced enzymatic changes in the liver of test rats (Rahman et al., 2002). Crude extracts of *S. maydis* is toxic to poultry (Blaney et al., 1981; Rabie et al., 1987) and Diplodiosis, a neuromycotoxicosis characterized by ataxia, paresis and paralysis, was induced in cattle, sheep and goats when dosed with culture extracts (Kellerman et al., 1985). Diplodiatoxin has furthermore been reported to cause mycotoxic peripheral myelinopathy and hepatitis in monkeys (Fincham et al., 1991). The fungus has primarily been isolated from maize in South Africa, South America and North America (Bensch and Vanstaden, 1992; Munkvold and Yang, 1995; Odriozola et al., 2005; Rheeder et al., 1995) and as it has not been isolated from European maize or cereals it cannot be regarded as a threat to cattle health in Denmark.

A species which is often associated with maize is *Epicoccum nigrum* (Lauren and Di Menna, 1999; Martyniyuk, 2003; Schumann et al., 1991a). *E. nigrum* is also a common contaminant of cereals in Denmark (Andersen et al., 1996) and it is therefore also likely to occur on Danish maize. *E. nigrum* is the only recognized species of its genus (Domsch et al., 2007) whose closest relatives are found within the *Phoma* genus (Arenal et al., 2000). The species can produce a wide range of secondary metabolites of which several have antimicrobial properties (Baute et al., 1978; Deffieux et al., 1978; Madrigal et al., 1991; Shu et al., 1997; Wright et al., 2003; Zhang et al., 2007). *E. nigrum* is also a strong producer of colored pigments, which have potential application prospects as natural food colorants (Mapari et al., 2005; Mapari et al., 2006; Mapari et al., 2008). Although *E. nigrum* is a very common contaminant of cereals and maize, it is still undetermined whether these agricultural commodities are contaminated with bioactive compounds produced by this species.

The genus *Phoma* is like *Epicoccum* also a common contaminant of maize world wide (Gonzalez et al., 2002; Ono et al., 2002; Schumann et al., 1991a). The genus *Phoma* includes more than 220 specific and intraspecific taxa (Boerema et al., 2004). The actual number of taxa within the genus *Phoma* is probably much higher, as only a fraction of the thousands of species described in literature have been verified *in vitro* (Aveskamp et al., 2008). The genus is a rich source of secondary metabolites and a search in the Antibase 2008 database (Laatsch, 2008) will return 282 metabolites of which more than 60% are from an identified *Phoma* species. Often when *Phoma* strain are isolated from maize or cereal samples they are not identified to species level, but simply listed as *Phoma* spp. It is therefore difficult to hypothesize which species and metabolites may be present in Danish maize.

An important *Phoma* species in maize is *Ph. maydis*. It has been reported as the causal agent of phaeosphaeria leaf spot (Carson, 1999), which is a severe foliar maize disease widely distributed in Central and South America, Asia, and Africa (Rane et al., 1966). The disease has the potential to cause great yield losses in United States of America (Carson, 2005). The metabolite profile of the causal agent, *Ph. maydis*, has unfortunately not be examined and it therefore unknown whether this species has the ability to produce mycotoxins during infections. Neither the disease nor the pathogen has been observed in Europe and it is therefore unlikely that Danish maize plants are infected with *Ph. maydis*. Another *Phoma* species, *Ph. sorghina*, has been associated with a disease with similar symptoms as phaeosphaeria leaf spot in Brazil (do Amaral et al., 2004; do Amaral et al., 2005). This is interesting because *Ph. sorghina*, like some *Alternaria* species, is reported as a producer of tenuazonic acid (Shephard et al., 1991). It has however not

been isolated from other *Phoma* species and it is unknown whether *Phoma* spp. may have contributed to the tenuazonic acid contamination observed in various surveys of wheat samples (Azcarate et al., 2008; Li and Yoshizawa, 2000; Webley et al., 1997).

RESULTS AND DISCUSSION

In order to get an idea of which mycotoxins may be present in Danish maize it is necessary to determine the mycobiota. Surveys of maize in neighboring countries have pointed at *Fusarium* and Dematiaceous fungi like *Alternaria, Epicoccum* and *Phoma* as the dominating mycotoxin producing genera. As there is a huge variation in metabolite profiles of individual species it is necessary to know which species are present in Danish maize in order to be able to estimate which mycotoxins are relevant to screen for.

FUSARIUM

ISOLATION AND IDENTIFICATION OF *FUSARIUM* SPECIES

To determine the occurrence of *Fusarium* in Danish maize plants 28 samples were collected at harvest 2005 and plated on the *Fusarium* selective medium Czapek-Dox iprodion dichloran agar (CzID) (Abildgren et al., 1987). The isolated *Fusarium* strains were grown on Yeast Extract Sucrose agar (YES) (Samson et al., 2004), potato dextrose agar (PDA) (Samson et al., 2004) and Spezieller Nährstoffarmer Agar (SNA) (Nirenberg, 1976) and identified to species level based on morphology (Sørensen et al., 2007) (**II**). One or more *Fusarium* species could be isolated from all of the 28 examined maize samples. The predominant species were *F. avenaceum* and *F. graminearum*, which both were present in 13 samples (**Figure 11**). *F. culmorum* and *F. equiseti* were also frequently isolated, occurring in 12 and 10 samples, respectively.

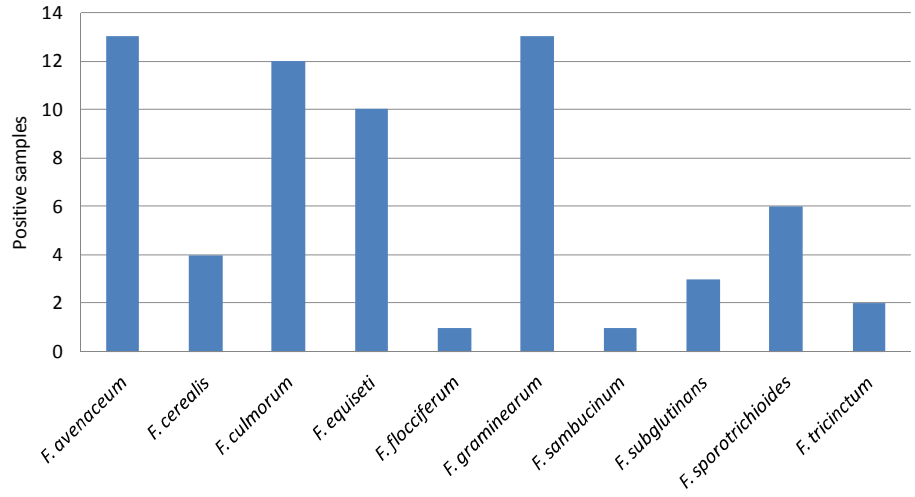


Figure 11. Occurrence of *Fusarium* species in 28 maize samples collected at harvest.

These results are generally in line with observations in cereals and maize in the neighboring countries (Andersen et al., 1996; Jestoi et al., 2004; Kosiak et al., 2003; Schumann et al., 1991b). The findings suggests that the most abundant *Fusarium* mycotoxins which may occur in Danish maize are type B trichothecenes and zearalenone (produced by *F. cerealis*, *F. culmorum* and *F. graminearum*), type A trichothecenes (produced by *F. equiseti* and *F. sporotrichioides*), moniliformin produced by (*F. avenaceum, F. subglutinans* and *F. tricinctum*), and beauvericin and enniatins (produced by *F. avenaceum, F. sambucinum, F. sporotrichioides, F. subglutinans* and *F. tricinctum*). The results indicate also that fumonisins are not

important mycotoxins in Danish maize, because the main producers, *F. verticillioides* and F. *proliferatum*, were not detected. As the occurrence of zearalenone, trichothecenes and fumonisins were examined by other project partners (Nielsen et al., 2005a; Nielsen et al., 2006; Nielsen et al., 2007; Nielsen et al., 2008), I decided to focus on mycotoxins, moniliformin, beauvericin and enniatins, which potentially also could be important.

OCCURRENCE OF MONILIFORMIN

In order to determine the occurrence of moniliformin in samples of whole maize plants a new method was needed, because existing methods were optimized for cereal or maize grains (Filek and Lindner, 1996; Jestoi et al., 2003; Kandler et al., 2002; Sulyok et al., 2006). Moniliformin is a small polar compound that is poorly retained on normal reverse phase columns, which are the most commonly used HPLC columns as they provide very sharp peaks and are compatible with atmospheric pressure ionization mass spectrometric (MS) techniques such as electrospray ionisation (ESI). Increased retention of moniliformin can be achieved using positively charged ion-pairing reagents (Filek and Lindner, 1996; Kandler et al., 2002; Munimbazi and Bullerman, 1998; Shepherd and Gilbert, 1986; Uhlig et al., 2004), although these may have some influence on MS detection efficiency. Using hydrophilic interaction chromatography (HILIC), we developed a method by which moniliformin could be successfully retained (**Figure 12**) (Sørensen et al., 2007) (**II**). HILIC had previously been used for detection of other highly polar compounds like carbohydrates, glycopeptides, nucleic acids, and shellfish toxins (Alpert, 1990; Dell'Aversano et al., 2005), but this was the first time it was used for detection of mycotoxins. The method was compatible with UV and MS detection with quantification limits of 96 ppb and 12 ppb, respectively.

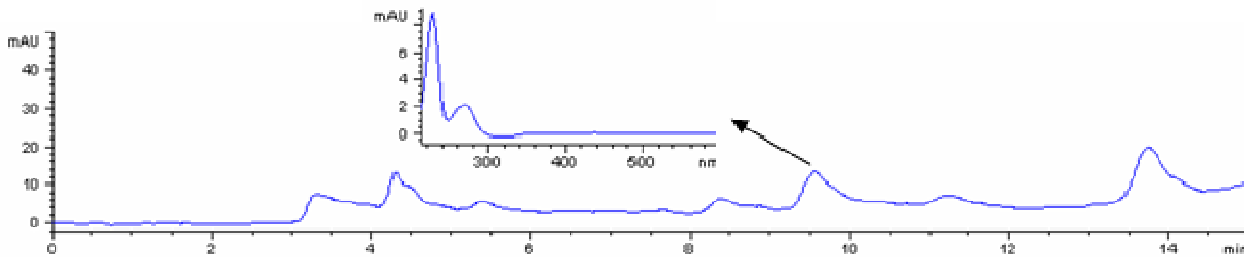


Figure 12. Chromatogram of a maize sample spiked with 288 ppb moniliformin with confirmation spectrum of moniliformin insert. Figure from Sørensen et al. (2007) (**II**).

28 maize samples collected at harvest 2005 were analyzed using HILIC with UV and MS detection and moniliformin was detected by MS in 15 of these samples. Moniliformin was however below limit of quantification (12 ppb) in all the positive samples, indicating that although moniliformin is a frequent contaminant of Danish maize, the levels can be assumed to be non-toxic to dairy cattle. The levels of moniliformin were quite lower than those found in Finish and Norwegian cereals where moniliformin was detected in levels up to 810 ppb (Jestoi et al., 2004) and 950 ppb (Uhlig et al., 2004), respectively. The levels were also lower than those previously found in surveys of maize kernels (Filek and Lindner, 1996; Sharman et al., 1991). Our samples were derived from whole maize plants and the low moniliformin levels can be

explained by at least two scenarios: i) the moniliformin producing fungi do not colonize the entire plants and the moniliformin amounts is therefore diluted by uninfected plant parts; ii) the moniliformin producing fungi can colonize the entire plants, but do not produce moniliformin when they grow on stems and leaves.

OCCURRENCE OF BEAUVERICIN AND ENNIATINS

To determine the occurrence of beauvericin and enniatin A, A1, B and B1 in samples of whole maize a LC-MS/MS method for simultaneous detection of these compounds was developed (Sørensen et al., 2008) (**III**). LC-MS/MS was the obvious choice because they ionize very well in positive electrospray due to their numerous heteroatoms. In our method the compounds were separated on a Gemini C6-Phenyl column (Phenomenex) by which all four enniatins could be separated (**Figure 13**). In most methods beauvericin co-elutes together with the predominant enniatin B or B1 (Jestoi et al., 2005; Sulyok et al., 2007; Uhlig and Ivanova, 2004), but in our method beauvericin co-eluted with the less frequently occurring enniatin A, which may enhance beauvericin sensitivity.

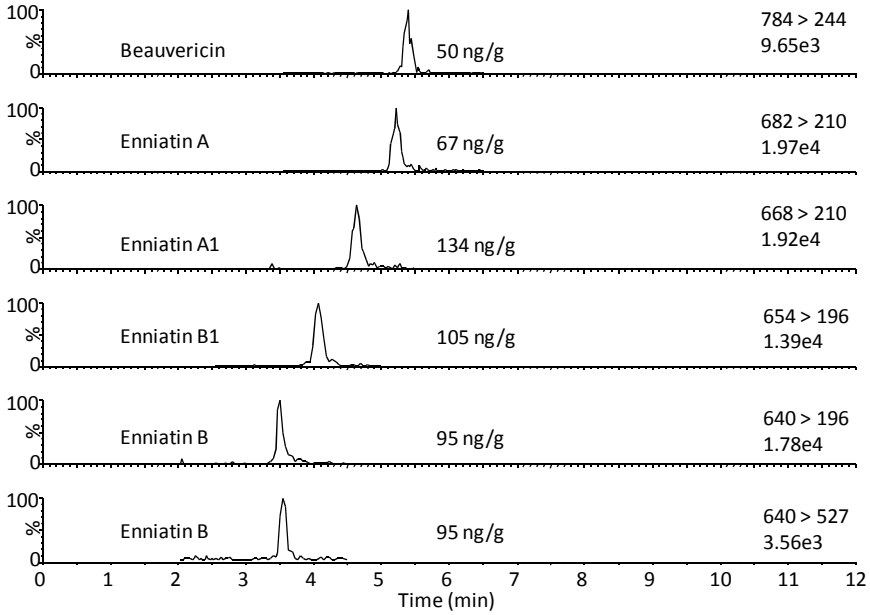


Figure 13. The extracted chromatograms of the quantifier ions from a fresh maize sample spiked with 50, 67, 134, 104 and 95 ng g^{-1} beauvericin and enniatins A, A1, B1 and B, respectively. The qualifier of enniatin B is also shown. Peak heights of the ion transitions are given as counts per second and %. Figure taken from Sørensen et al. (2008) (**III**).

The method was used to analyze 30 and 43 samples derived from whole maize plants collected at harvest 2005 and 2006, respectively (**Table 2**). All samples had the same distribution of the enniatins: B > B1 > A1 > A. Enniatin B was present in 90% of the samples in 2005 and in 100% in 2006 at levels up to 489 and 2598 ng g^{-1}, respectively. Beauvericin contamination was more frequently detected in 2006 than in 2005 (89 and 10%, respectively) and in higher amounts (988 and 71 ng g^{-1}, respectively). The difference in beauvericin and enniatin contamination between the two years may be caused by climatic differences. The summer and autumn were warmer and wetter in 2006 than in 2005 (Statistics Denmark, 2006; Statistics Denmark, 2007), which may have enhanced *Fusarium* infections.

Table 2. Occurrence and content of enniatins and beauvericin in whole maize in 2005 and 2006 at harvest. Data for maize grain samples from the harvest 2006 are also shown. Table deduced from Sørensen et al. (2008) (**III**).

		n[a]	Positive (%)	Mean (ng g^{-1})	Median (ng g^{-1})	Range (ng g^{-1})
2005	Enniatin A	30	3	0	nd[b]	nd – <17
	Enniatin A1	30	10	1	nd	nd – <34
	Enniatin B	30	90	124	75	nd – 489
	Enniatin B1	30	47	9	nd	nd – 79
	Beauvericin	30	10	4	nd	nd – 73
2006	Enniatin A	43	12	6	nd	nd – 106
	Enniatin A1	43	35	13	nd	nd – 107
	Enniatin B	43	100	366	204	<24 – 2598
	Enniatin B1	43	84	81	44	nd – 496
	Beauvericin	43	98	116	32	nd – 988

To examine whether enniatins and beauvericin could also be detected in silage 20 samples collected from three months old silage stacks were analyzed. The samples contained less beauvericin and enniatins than the fresh maize samples, with enniatins A and A1 being absent. As noted before, enniatin B was the predominant compound, occurring in 95 % of the samples, ranging up to 218 ng g^{-1}. The stability of enniatin B was examined in 10 silage stacks by analyzing samples collected in 3-, 7-, and 11-month-old silage stacks (**Figure 14**). The results show that enniatin B is very stable in the silage stacks and was present in all stacks after 11 months. The results did not show a consistent trend: three locations had the highest amounts after 3 months, three locations after 7 months, and four after 11 months. Samples from stacks 1 and 15 contained high amounts of enniatin B at all time points, whereas samples from stacks 4, 16, 17, and 19 contained low amounts of enniatin B. The average and median showed a small increase in enniatin B as the silage got older. These results suggest that the enniatins are not degraded in the period tested.

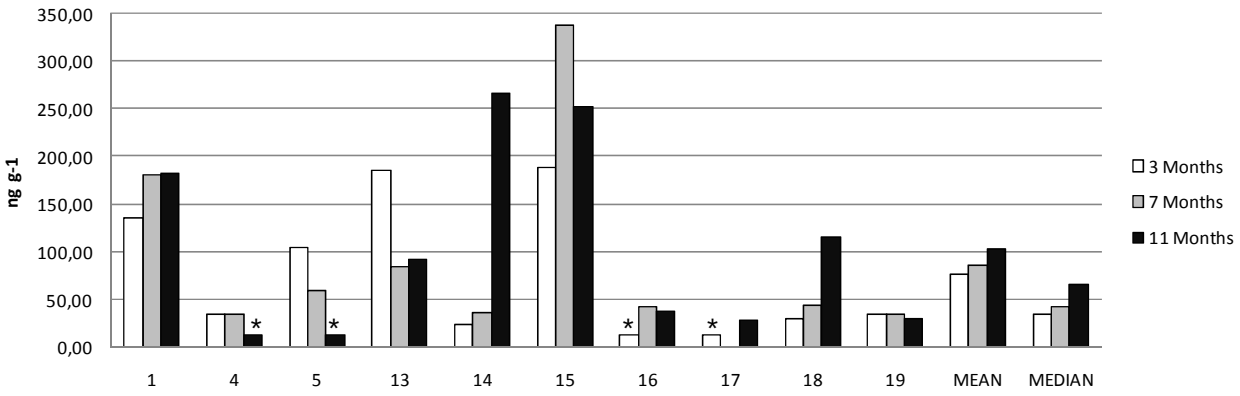


Figure 14. Content of Enniatin B in 10 different maize silage stacks collected after respectively 3, 7 and 11 months of ensiling. *Trace amounts below LOQ. Means are calculated by setting samples in which the compounds were not detected to 0 and trace values below LOQ to LOD. Figure from Sørensen et al. (2008) (**III**).

Lactic acid bacteria, which are responsible for the ensiling process, have been shown to be able to bind or transform other *Fusarium* mycotoxins such as trichothecenes, zearalenone, and fumonisins (Niderkorn et al., 2006; Niderkorn et al., 2007). The lactic acid bacteria are mainly active in the first months of the ensiling

process, where they may have transformed some of the enniatins, which explains why the levels were lower in three months old silage than in fresh maize.
The results of the surveys of moniliformin, enniatins and beauvericin showed that the levels in Danish maize are low even though they may occur quite frequent. As this was also the case for DON, NIV, T-2, HT-2, ZEA and fumonisins (Nielsen et al., 2005a; Nielsen et al., 2006; Nielsen et al., 2007; Nielsen et al., 2008) it seems unlikely that *Fusarium* mycotoxins have played a crucial role in the problems observed at dairy cattle farms in Denmark.

DEMATIACEOUS FUNGI

The heterogeneous group of dematiaceous fungi comprises a wide range of fungi producing an olive-grey, brown or black pigment (melanin) in the cell wall of most hyphae or conidia. A pre-study showed that the most frequently occurring dematiaceous fungi occurring in Danish maize were *Alternaria, Epicoccum* and *Phoma*. The following sections describe the isolation and characterization of some of the most important Dematiaceous fungi.

ISOLATION AND IDENTIFICATION OF *ALTERNARIA*, *EPICOCCUM* AND *PHOMA*

Isolation of dematiaceous fungi like *Alternaria, Epicoccum* and *Phoma* often performed with standard media like dichloran Rose Bengal yeast extract sucrose agar (DRYES) (Frisvad, 1983), dichloran 18% glycerol agar (DG18) (Hocking and Pitt, 1980), V8 juice agar (V8) (Simmons, 1992). Isolation of *Alternaria, Epicoccum* and *Phoma* is however often hampered by other competing fungi like *Aspergillus*, *Fusarium*, *Penicillium* and various Zygomycetes when using these media, especially in complex commodity like samples derived from whole maize plants. Enumeration of the three target genera can therefore be underestimated and more appropriate isolation media are therefore desirable. Previously a semi-selective medium for isolation of dematiaceous hyphomycetes, including small spored *Alternaria* was developed (Andrews and Pitt, 1986). Although this medium reportedly reduced the growth of *Aspergillus*, *Penicillium* and Zygomycetes, these fungi would still emerge in samples where they dominated over *Alternaria*. Another media based on acidified weak potato-dextrose agar (AWPDA) with thiabendazole for isolation of small spored *Alternaria* has been developed (Hong and Pryor, 2004). The medium does however not reduce growth of Zygomycetes, which due to their fast growing ability are among the most troublesome groups when isolating Dematiaceous fungi, including *Alternaria*. Because these two media have some minor drawbacks, a need for a new isolation medium for isolation of Dematiaceous fungi still exists. We therefore set out to develop a new medium for isolation of *Alternaria, Epicoccum* and *Phoma* and after having tried various fungicides and heavy metals we ended up with a medium containing potato carrot agar with manganese (PCA-Mn) (Sørensen et al., 2009b) (**IV**). The medium successfully inhibited growth of *Aspergillus, Cladosporium, Penicillium,* various Zygomycetes and several *Fusarium* species except *F. verticillioides* and *F. oxysporum*. The usefulness of PCA-Mn to isolate selected dematiaceous fungi is illustrated in **Figure 15**, where barley and wheat grains are placed on V8, DG18, DRYES and PCA-Mn. Various undesired genera including *Aspergillus, Eurotium, Fusarium* and *Mucor*, emerged from the grains on V8, DG18 and DRYES. They did however not emerge on PCA-Mn where only *Alternaria infectoria* and *Epicoccum nigrum,* in addition to a *Bipolaris* sp., were isolated.

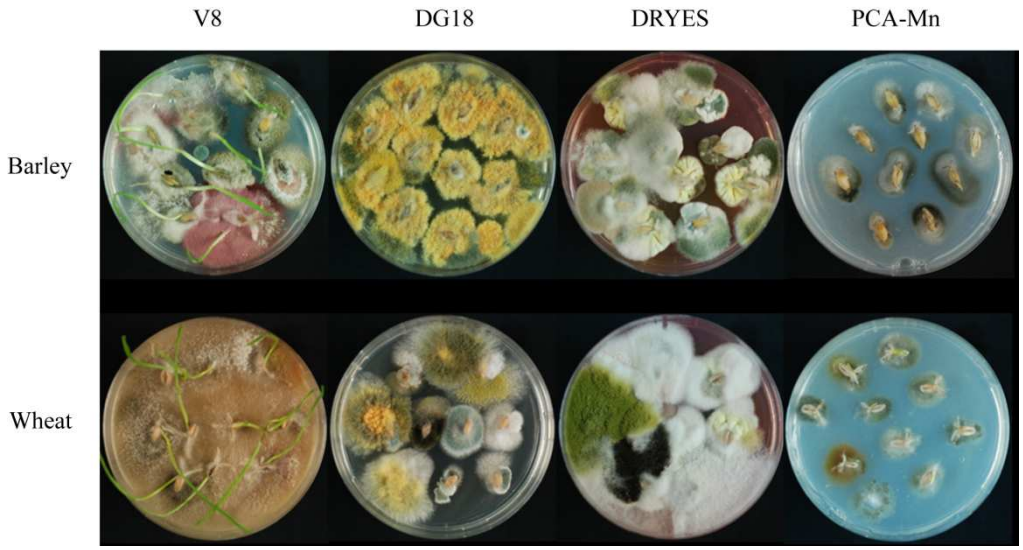


Figure 15. Barley and wheat grains incubated on V8, DG 18, DRYES and PCA-Mn for one week. Figure taken from Sørensen et al. (2009b) (**IV**).

The medium was successfully used to isolate *Alternaria, Epicoccum* and *Phoma* from maize collected at harvest. The distribution of 27 *Alternaria* strains isolated from 40 maize samples was: *A. arborescens* species-group (3), *A. infectoria* species-group (15) *and A. tenuissima* species-group (9) (unpublished results). This distribution concurred with previous studies of Danish malt barley (Andersen et al., 1996) and Norwegian cereal grains (Kosiak et al., 2004), which both identified the *A. infectoria* species-group and the *A. tenuissima* species-group as dominating small spored *Alternaria*. In two surveys of ensiled maize in Germany, *A. alternata* was identified as the predominant species (Müller, 1991; Schumann et al., 1991a). Members of the *A. infectoria* species-group were only rarely identified in these two surveys, but there is however a risk that they have been misidentified as *A. alternata*, which is a common occurring error (Simmons, 2002).

27 *Phoma* strains and 25 *Epicoccum* strains were also isolated from 40 maize samples using PCA-Mn (unpublished results). All the isolated *Phoma* strains were identified as *P. pomorum*, whereas all *Epicoccum* strains were identified as *E. nigrum*, which is the only recognized *Epicoccum* species (Domsch et al., 2007). Several studies have previously identified *E. nigrum* as a common contaminant of maize (Lauren and Di Menna, 1999; Macek and Zupan, 1993; Martyniyuk, 2003; Schumann et al., 1991a), but to my knowledge this is the first time *P. pomorum* has been identified as a major contaminant of maize. Usually when *Phoma* strains have been isolated from maize they have not been identified to species level (Ono et al., 2002; Schumann et al., 1991a) and it is therefore possible that strains of *P. pomorum* have been isolated before.

After having established that the *A. infectoria* species-group and *P. pomorum* are frequent contaminants of Danish maize they were characterized in two separate studies, both using polyphasic approaches. As part of these studies the metabolite profiles of the two species were examined, which could be used to identify compounds that may be produced in maize plants and thereby pose a risk to dairy cattle.

POLYPHASIC CHARACTERIZATION OF THE *ALTERNARIA INFECTORIA* SPECIES-GROUP

Eleven representative strains isolated from maize were included in a polyphasic study of the *A. infectoria* species-group (Andersen et al., 2009) (**V**). One of the major overall aims of this study was to examine the

taxonomical relationship between members of the species-group using phylogenetic analyses, metabolite profiles and morphological characteristics. A personal aim was to examine whether the strains isolated from Danish maize differed from members of the species-group isolated from other sources and parts of the world. The hypothesis before the study started was that members of the *A. infectoria* species-group were sexual fungi and that the polyphasic approach would reveal a number of new naturally occurring species. To test this hypothesis eleven *A. infectoria* strains isolated from Danish maize were included in the study together with other strains of *A. infectoria* in addition to *A. arbusti*, *A. ethzedia*, *A. intercepta, A. metachromatica, A. oregonensis*, *A. photistica, A. triticimaculans, A. triticina* and *A. viburni*, which are also members of the species-group. Three genes commonly used in phylogenetic studies and species differentiation, internal transcribed spacer region (ITS), translation elongation factor 1α (TEF-1α) and glyceraldehyde-3-phosphate dehydrogenase (GPD), yielded nearly identical sequences for all members of the *A. infectoria* species-group. Metabolite analysis of strains grown on DRYES showed that all members of the *A. infectoria* species-group produced infectopyrone and 4z-infectopyrone, whereas most strains produced novae-zelandin A and B (**Figure 16**). Several strains produced also derivatives of altertoxin, a compound normally produced by *A. alternata, A. arborescens* and *A. tenuissima* (Andersen et al., 2002). The results of the study showed that although members of the species group could be differentiated from the related species *Embellisia abundans, Chalastospora cetera* and *A. malorum* by chemical classification, molecular cladification and morphological characterization, these three methods were not sufficient to discriminate strains within the species-group. The results did therefore not support the initial hypothesis; instead the study indicated that members of the species-group are homothallic and/or clonal, which means that artificial identification systems based on any stable phenotypical differentiation characters still are needed to detect, recognize and identify these fungi.

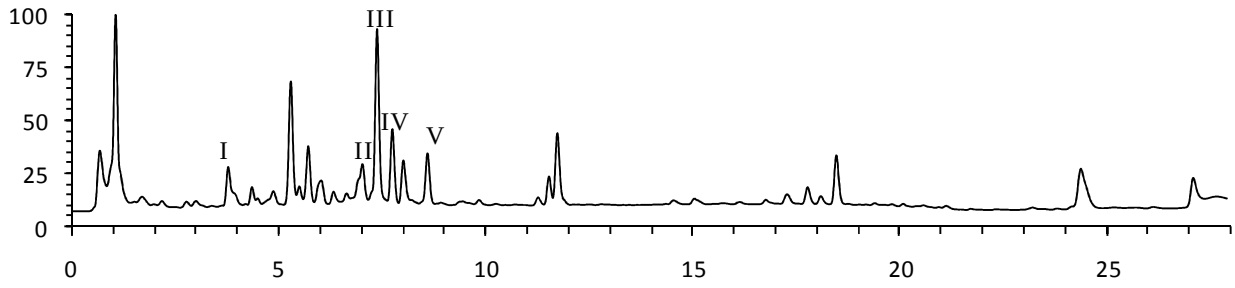


Figure 16. HPLC chromatogram at 210 nm of *A. infectoria* (BA 1765) grown on DRYES used in Andersen et al. (2009) (**V**). Peaks of novae-zelandin A (I), 4z-infectopyrone (II), infectopyrone (III), altertoxin derivative (IV) and novae-zelandin (V) are indicated.

POLYPHASIC CHARACTERIZATION OF *PHOMA POMORUM*

To characterize *P. pomorum* isolated from Danish maize 22 strains were included in a polyphasic study together with isolates representing several other species of the *Phoma* section *Peyronellaea* (Sørensen et al., 2009a) (**VI**). ITS sequence analysis grouped Danish *P. pomorum* strains together with *P. pomorum* reference strains in a cluster separated from the other species. All *P. pomorum* strains had nearly identical ITS sequences with only one or two mutations. For metabolite analysis all *Phoma* strains were grown on DRYES, as this medium supported production of most metabolites and in highest amounts. As observed in the phylogenetic analysis *P. pomorum* grouped separately from the other species when analyzing

metabolite production on DRYES. The metabolite analysis showed that *P. pomorum* produced several compounds with similar UV spectra, which are typical for the group of isocoumarins (**Figure 17**). Isocoumarins are structurally related compounds with a 1H-2-benzopyran-1-one backbone and alternating side groups produced by plants, bacteria and fungi (Barry, 1964). Isocoumarins have a wide range of biological activities; some are toxic to animals including humans (Marquardt and Frohlich, 1992), whereas others have antimicrobial (Okazaki et al., 1975), mutagenic (Varanda et al., 1997) or antitumor properties (Kuhr et al., 1973).

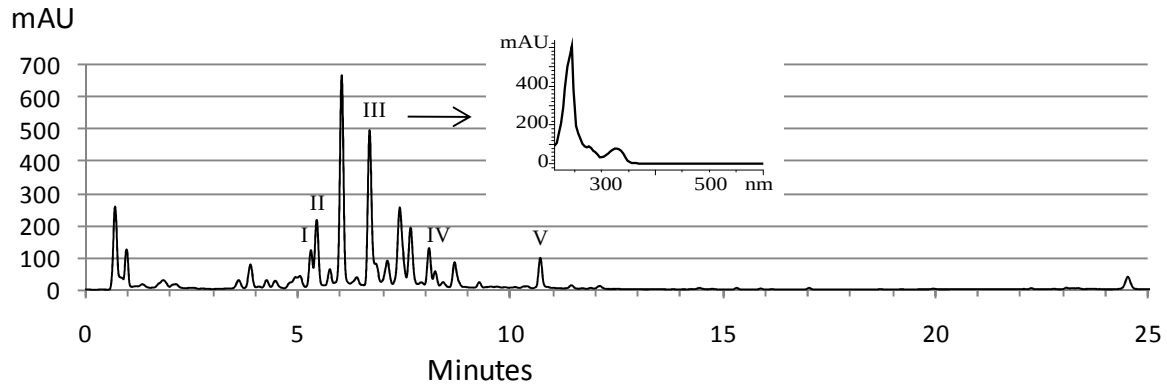


Figure 17. HPLC-UV-VIS chromatogram of *P. pomorum* (IBT 41377) at 246 nm. Peaks of citreo-isocoumarinol (I), citreo-isocoumarin (II), diaportinic acid (III), diaporthin (IV) and dichlorodiaporthin (V) are indicated. Extracted spectrum of diaportinic acid has been inserted. Figure from Sørensen et al. (2009a) (**VI**).

The predominant isocoumarin produced by *P. pomorum* was identified as diaportinic acid, but it was also able to produce diaportinol, diaporthin, dichlorodiaporthin, citreoisocoumarin, 6-methyl citreoisocoumarin and citreoisocoumarinol (**Figure 18**).

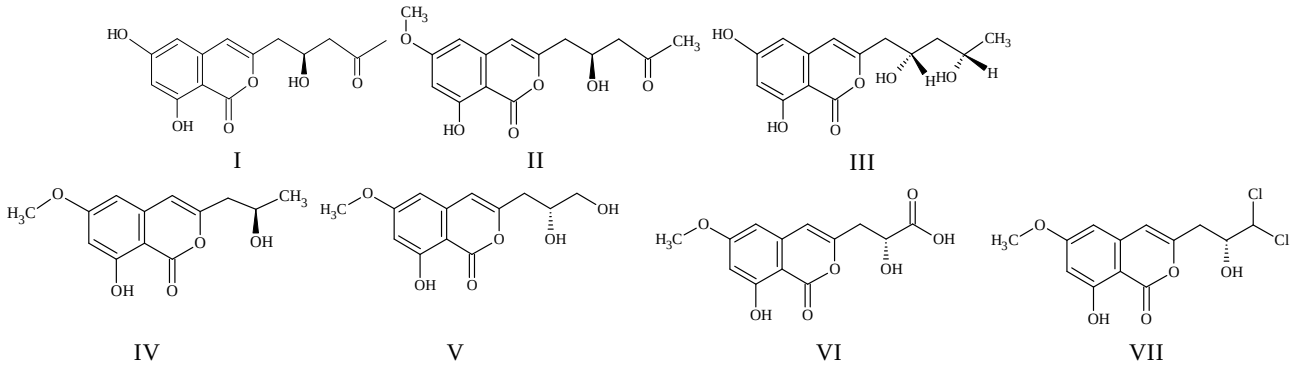


Figure 18. Structures of isocoumarins produced by *P. pomorum*. (I) citreo-isocoumarin, (II) 6-methyl citreo-isocoumarin, (III) citreo-isocoumarinol, (IV) diaporthin, (V) diaportinol, (VI) diaportinic acid, (VII) dichlorodiaporthin.

Diaportinic acid, diaportinol, diaporthin and dichlorodiaporthin have previously only been reported from *Penicillium nalgiovense* (Larsen and Breinholt, 1999), whereas citreoisocoumarins have been isolated from several species of *Penicillium* and *Aspergillus* (Lai et al., 1991; Larsen and Breinholt, 1999; Malmstrom et al., 2000; Watanabe et al., 1998). Currently, there is no existing knowledge on the biological activity of the

isocoumarins produced by *P. pomorum* and how the presence of these compounds will affect dairy cattle if they are produced in Danish maize.

DETECTION OF *ALTERNARIA* AND *PHOMA* METABOLITES IN MAIZE

As previously shown *A. infectoria* isolated from Danish maize is able to produce a wide range of metabolites including infectopyrones, novae-zelandins and altertoxin derivatives on the artificial growth medium DRYES. On the same medium *P. pomorum* produced several isocoumarins, with diaportinic acid as the predominant analogue. An artificial medium made from pieces derived from whole maize plants was prepared and the metabolite production of the two species was examined by HPLC-UV in order to assess if they are able to produce some of their metabolites on maize (experiments described in **Appendix 2**). The results showed that *A. infectoria* was able to produce infectopyrones and novae-zelandins, whereas the altertoxin derivatives could not be detected. *P. pomorum* produced diaportinic acid on the artificial maize medium as well as small amounts of a few other isocoumarins (**Figure 19**).

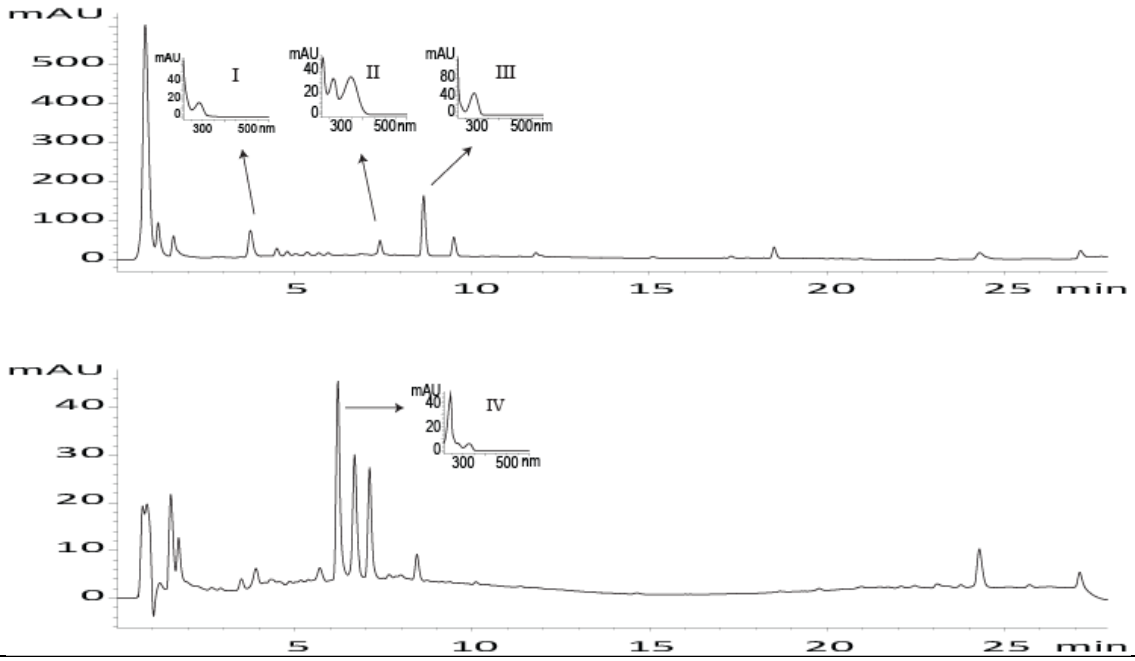


Figure 19. HPLC-UV chromatograms of *Alternaria infectoria* (top) and *Phoma pomorum* (bottom) collected at 210 nm and 246 nm, respectively. Extracted confirmation spectra of novae-zelandin A (I), infectopyrone (II), novae-zelandin B (III) and diaportinic acid (IV) have been inserted.

Although the metabolite production of the two species was lower on the maize medium than on DRYES, the results indicate that they may be able to produce some of their metabolites on living maize. To examine whether some of these metabolites can be detected in maize collected at harvest an LC-MS/MS method for detection of infectopyrones and diaportinic acid was developed (described in **Appendix 2**, **Figure 20**). With this method at least four infectopyrone analogues could be detected in *A. infectoria* extracts with the used settings, indicating the presence of at least two extra analogues in addition to infectopyrone and 4z-infectopyrone. The method was optimized on extracts of *A. infectoria* and *P. pomorum* and then used to screen ten randomly selected maize samples.

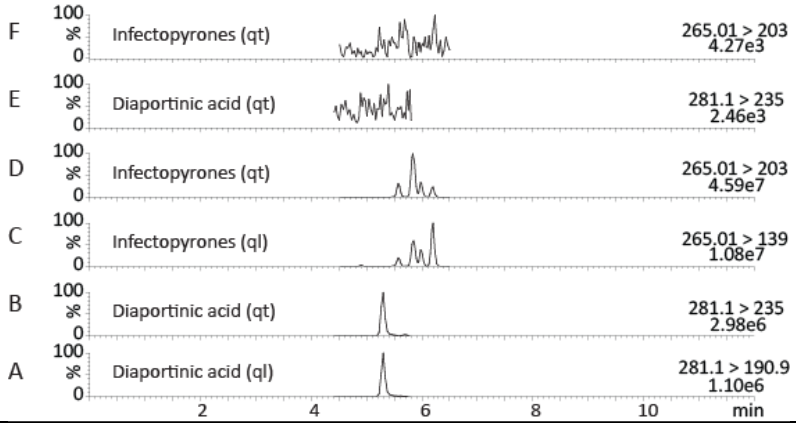


Figure 20. LC-MS/MS detection of quantifier (qt) and qualifier (ql) ions of diaportinic acid (A-B) and infectopyrones (C-D) in percent and in counts per second detected in extracts from *Phoma pomorum* and *Alternaria infectoria*, respectively. Chromatograms of qt ions of the two compounds from a maize sample is also included (E-F).

As the limits of detection and quantification of diaportinic acid and infectopyrones in the maize samples were not determined this experiment was mainly used to determine whether diaportinic acid and infectopyrone were present in high amounts hypothesizing that high levels would be detected. Neither diaportinic acid nor infectopyrones were however detected in any of the ten samples and further experiments were therefore abandoned. This then leaves the conclusion that diaportinic acid and infectopyrones compounds are most likely not present in high amounts in maize and they are therefore possibly not a threat to Danish dairy cattle.

ADDITIONAL PROJECTS

During my PhD I got involved in two additional side projects, which both dealt with areas where *Fusarium* species cause severe economical problems for farmers and cause great health concerns for consumers. The first side project concerned the emerging disease wet apple core rot in collaboration with Dr. Hans-Josef Schroers from Agricultural Institute in Slovenia and the second side project concerned the occurrence of the *Gibberella fujikuroi* species complex in rice in collaboration with Dr. Ednar Wulff from the Danish Seed Health Centre.

WET APPLE CORE ROT

While working with *F. avenaceum* metabolites in maize I got involved in a project on wet apple core rot (wACR), which is a new emerging apple disease in Slovenia with *F. avenaceum* as the causal agent (Schroers et al., 2008). The disease starts with white, rose, or reddish mycelium developing initially in the apple core, which then spreads destructively into the surrounding cortex of infected apples as a light-brown wet rot (**Figure 21**).

Figure 21. Early (left) and late (right) symptoms of wet apple core rot. Picture from Sørensen et al (2009c) (**VII**).

In a survey of 21 Gloster apple trees in Slovene orchards in the fall of 2004, ca. 5% of the apples showed symptoms of wACR, and wACRs were also regularly encountered in other cultivars, such as Fuji and Jona Gold in 2004-2006 (Schroers et al., 2008). A LC-MS/MS method for simultaneous detection of thirteen of the most important *F. avenaceum* metabolites was developed to examine their occurrence during wACR (Sørensen et al., 2009c) (**VII**). The metabolites included in the method were moniliformin, acuminatopyrone, chrysogine, chlamydosporol, antibiotic Y, 2-amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol), aurofusarin, and enniatins A, A1, B, B1, B2, and B3 and using the Gemini C6-Phenyl column they eluted with little or no overlap (**Figure 22**). Levels of moniliformin, antibiotic Y, aurofusarin and enniatins A, A1, B and B1 were quantitatively examined with this method with quantification limits of 50, 50, 50, 17, 34, 24 and 26 ppb. To achieve a quantitative detection of aurofusarin, we had to make our own reference standard of this compound because it is not commercially available. The remaining compounds, chrysogine, chlamydosporol, antibiotic Y, 2-AOD-3-ol and enniatins B2 and B3 could only be assessed in relative amounts due to insufficient amounts of metabolite standards.

Five *F. avenaceum* strains were artificially inoculated to apples after which metabolites were extracted after 3, 7, 14 and 21 days of incubation. Most metabolites were detected after 3 or 7 days and reached significantly high levels within 14 or 21 days (Sørensen et al. 2009c) (**VII**). The highest levels of moniliformin,

antibiotic Y, aurofusarin, and the combined sum of enniatins A, A1, B, and B1 were 7.3, 5.7, 152, and 12.7 ppm, respectively. Interestingly, the highest amounts of moniliformin, aurofusarin, 2-AOD-3-ol, and chrysogine were measured in the majority of samples already after 14 days, which may indicate that the metabolism rate of the strains decreases or comes to a steady state after a certain amount of time.

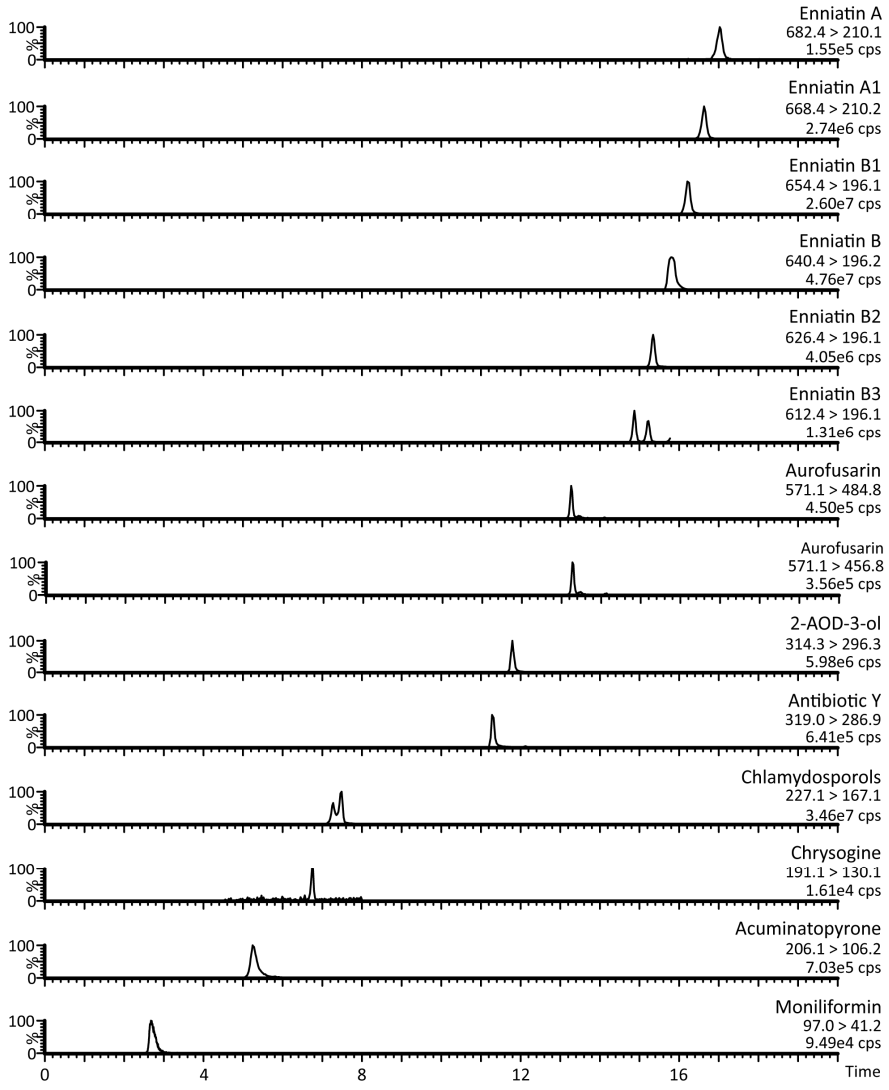


Figure 22. LC-MS-MS detection of quantifier ions of 13 metabolites from a *F. avenaceum* strain on YES. Qualifier ion of aurofusarin is included. Figure taken from Sørensen et al. (2009c) (**VII**).

To determine the occurrence of the thirteen metabolites under natural conditions twenty apples showing wACR symptoms were collected and analyzed (**Table 3**). One or several metabolites could be detected in seventeen samples, with aurofusarin and antibiotic Y occurring most frequently and in highest amounts. The maximum levels of antibiotic Y and aurofusarin were 51 ppm and 145 ppm, respectively, whereas the maximum levels of moniliformin and the combined sum of enniatin A, A1, B and B1 were 2.9 ppm and 3.9 ppm, respectively. 2-AOD-3-ol and chrysogine were also frequently observed and they were detected in 11

and 10 samples, respectively. Chlamydosporols and acuminatopyrone co-occurred in four samples, suggesting that these two structurally related compounds share the same regulation mechanism.

Table 3. Occurrence of moniliformin (mon), antibiotic Y (anti Y), aurofusarin (auro) and combined sum of enniatins (Σ enn) (μg/g) and 2-AOD-3-ol, chrysogine (chrys), chlamydosporols (chlam) and acuminatopyrone (acumi) (%) in 20 naturally infected apples sampled from trees [a]. Table from Sørensen et al. (2009c) (**VII**).

	ppm				% [b]			
#	mon	anti Y	auro	Σ enn	2-AOD-3-ol	chrys	chlam	acumi
1	1.28		13.2	0.13	0.4	45.2	27.4	63.4
2								
3								
4	trace	2.57	0.64	0.34	0.4			
5	0.25	9.68	74.4	1.63	24.7	44.7		
6	2.87	trace	80.7	trace		12.9		
7	0.20	trace	145.1		4.3	11.3		
8	trace	0.29	34.4	0.12	2.1			
9		trace						
10	2.53	1.55	128.1	3.89	0.6	17.5		
11	2.74	4.17	166.6	0.54	24.0	3.0		
12	0.34	7.60	63.8	0.16	7.1	2.8	25.8	89.8
13		trace	0.18					
14	0.16	17.1	95.4	1.65	11.3	29.5	100	100
15	1.53	0.11	91.6	0.31	1.5	100		
16	0.33	0.08	1.20	0.20		25.4	62.6	3.2
17	1.32	51.3	103.6	2.60	100	21.0		
18	2.33	0.32	132.5	0.16	4.8			
19								
20								

[a] *Trace: below Limit of quantification.*
[b] *Highest peak area of each metabolite set to 100%.*

The results of this project show that *F. avenaceum* is able to produce a wide array of metabolites in high levels during wACR. Because the disease starts inside the apples infected apples can appear symptomless on the outside and there is therefore a risk that infected apples may be used in apple products like cider and juice. The disease therefore poses a health safety risk for consumers due to the high metabolite levels produced.

CHARACTERIZATION OF MEMBERS OF THE *GIBBERELLA FUJIKUROI* SPECIES COMPLEX ISOLATED FROM RICE

In collaboration with the Danish Seed Health Centre the metabolite production, genetic diversity and pathogenecity of strains of the *Gibberella fujikuroi* species complex isolated from Asian and African rice were examined (Wulff et al., 2009) (**VIII**). *F. fujikuroi* causes the disease bakanae (foolish seedling) in rice with the typical symptoms being slender, chlorotic and elongated primary leaves which are induced by the production of gibberellin by the pathogen (Amoah et al., 1995). Several other members of the species complex, such as *F. proliferarum* and *F. verticillioides* are also common pathogens of rice but do not

cause the same symptoms as *F. fujikuroi* as they do not produce gibberellins (Malonek et al., 2005). In addition to affecting crop yield, strains of the *G. fujikuroi* species complex are also a problem for consumers due to production of a number of mycotoxins including fumonisins, moniliformin, beauvericin and fusaproliferin (Desjardins et al., 2000; Reynoso et al., 2004). At present, there is limited information on occurrence and population structure of *G. fujikuroi* species complex associated with rice seeds originating from Africa and Asia. To provide more information on this subject the side project had four objectives: i) to determine incidence and severity of *G. fujikuroi* populations associated with rice seeds in Africa and Asia; ii) to assess species composition and genetic variability of the isolated strains using translation elongation factor 1-alpha (TEF-1α) DNA sequences; iii) to determine the ability of the strains to produce fumonisins and gibberellin A3 and iv) to correlate the obtained molecular and mycotoxin profiles with the pathogenicity of *G. fujikuroi* species on rice. *F. verticillioides* was the most frequently isolated species followed by *F. fujikuroi* and *F. proliferatum* and a group of unidentified isolates. All the isolated strains were pathogenic to rice and especially *F. fujikuroi* caused severe symptoms. Phylogenetic analysis of TEF-1α generated a tree consisting of four separate clades (I-IV) representing *F. verticillioides* (I), *F. fujikuroi* (II), *F. proliferatum* (III) and *Fusarium* spp. (IV). Metabolite profiling showed that only *F. fujikuroi* produced gibberellins (**Table 4**), confirming previous findings (Malonek et al., 2005).

All strains produced fumonisin B1, although strains of *F. fujikuroi* and *Fusarium* spp. only produced trace amounts. Several other fumonisin analogues, most notably B2, B3 and A1, were also detected again with *F. verticillioides* and *F. proliferatum* as the chief producers. All strains also produced fusaric acid and beauvericin, whereas *F. fujikuroi* was the only species which consistently produced fusarin A and C. Moniliformin was produced by most strains of *F. fujikuroi* and *F. proliferatum*, whereas none of the *F. verticillioides* strains produced this compound. The only reported moniliformin producing *F. verticillioides* strains are therefore still from banana (Moretti et al., 2004). These results show that strains isolated from rice are able to produce a wide range of mycotoxins and if they can also produce these compounds during infections they represent a risk for consumers of contaminated rice. Previously, fumonisins have been detected in rice in America (Abbas et al., 1998) and Asia (Park et al., 2005; Tanaka et al., 2007), but our results suggest that screening for other metabolites like moniliformin and beauvericin may be needed.

Table 4. Qualitative detection of moniliformin (MON), fusaric acid (FUS), gibberellin A3 (GA3), Fusarin A and C (FA and FA), fusaproliferin (FUP), beauvericin (BEA) and fumonisin B1 of *F. fujikuroi*, *F. proliferatum*, *F. sp.*, *F. verticillioides* and *F. subglutinans* on YES, RC and/or PDA. Data derived from Wullf et al. (2009) (**VIII**) and **Appendix 3**.

Species	No.	MON	FUS	GA3	FA	FC	FUP	BEA	FB1
F. fuj	7		X	X	X	X	X	X	X
F. fuj	20	X	X	X	X	X	X	X	X
F. fuj	21	X	X	X	X	X		X	X
F. fuj	26		X	X	X	X		X	X
F. fuj	28		X	X	X	X	X	X	X
F. fuj	29	X	X	X	X	X	X	X	X
F. fuj	33	X	X	X	X	X	X	X	X
F. fuj	37	X	X	X	X	X	X	X	X
F. fuj	49	X	X	X	X	X	X	X	X
F. fuj	55		X	X	X	X	X	X	X
F. pro	24	X	X				X	X	X
F. pro	34		X		X	X	X	X	X
F. pro	41	X	X				X	X	X
F. pro	51	X	X		X	X	X	X	X
F. pro	54		X			X	X	X	X
F. pro	57		X		X	X	X	X	X
F. sp.	1		X					X	X
F. sp.	38		X		X	X	X	X	X
F. sp.	44	X	X		X	X		X	X
F. sp.	53	X	X					X	X
F. ver	22		X					X	X
F. ver	23		X					X	X
F. ver	25		X				X	X	X
F. ver	31		X		X	X	X	X	X
F. ver	32		X					X	X
F. ver	35		X		X	X		X	X
F. ver	36		X		X	X		X	X
F. ver	39		X		X	X		X	X
F. ver	46		X		X	X		X	X
F. ver	47		X				X	X	X
F. ver	52		X					X	X
F. ver	53,2		X					X	X
F. ver	56		X					X	X

CONCLUSIONS

Danish maize samples collected at harvest were found to be highly infected with *Fusarium* as all 28 examined samples contained one or more *Fusarium* species. The predominant species were identified as *F. avenaceum*, *F. culmorum, F. graminearum* and *F. equiseti*, but several other species were also isolated. These findings helped to identify trichothecenes, zearalenone, moniliformin and enniatins as the most important *Fusarium* derived mycotoxins in Danish maize. While other project partners determined the occurrence of trichothecenes, zearalenone and fumonisins I developed novel methods for detection of moniliformin and enniatins. The occurrence of moniliformin was detected using HILIC with UV and MS, which was the first time this method was used for detection of mycotoxins. Enniatins and beauvericin were detected with LC-MS/MS, which was the first time that these compounds were detected in samples from whole maize plants. Although the examined maize samples were found to be frequently contaminated with one or more of the different mycotoxins, the levels were considered to be relatively harmless to dairy cattle feeding on maize silage.

To determine the occurrence of Dematiaceous fungi in maize a semi-selective medium (PCA-Mn) was developed. Using this medium *A. infectoria* was the most frequently isolated *Alternaria* species followed by *A. tenuissima* and *A. arborescens*. Characterization of the *A. infectoria* strains isolated from maize showed that they were similar to strains isolated from other sources and could not be differentiated by morphology, DNA sequence analysis or metabolite profiling. *A. infectoria* produced infectopyrones, novae-zelandins and altertoxin derivates on DRYES, but the strains produced however only small amount of infectopyrones and novae-zelandins on an artificial maize medium.

P. pomorum was the only isolated *Phoma* species and metabolite profiling showed that the isolated strains produced several isocoumarins with diaportinic acid as the predominant analogue. The metabolite profile of *P. pomorum* could be used to differentiate this species from other species of the *Phoma* section *Peyronellaea*.

A LC-MS/MS method was developed to screen maize samples for the occurrence of infectopyrones and diaportinic acid. Ten samples were examined using this method, but neither infectopyrones nor diaportinic acid was detected in any of the samples.

Based on the results obtained in my PhD it seems likely that the health problems that have occurred in Danish dairy farms are not caused by mycotoxins produced while the plants were growing in the fields. If mycotoxins are the cause of these problems they are therefore produced during the ensiling process of maize, where *Aspergillus*, *Penicillium, Byssochlamys* and *Monascus* are the predominant mycotoxin producing genera.

During my PhD I was involved in two projects concerning areas where *Fusarium* is a problem and where their mycotoxins represent a potential risk for consumers. In the first project an LC-MS/MS method for simultaneous detection of 13 *F. avenaceum* metabolites was developed and used in a survey of apples suffering of wet apple core rot. This disease in an emerging disease in Slovene orchards and the high observed levels of *F. avenaceum* metabolites can be a threat for consumers of apple products. Contaminated apples can easily end up in various products like juice or cider as the disease starts from the inside and infected apples can therefore appear symptomless on the outside. The second project concerned characterization of strains of the *Gibberella fujikuroi* species complex isolated from rice, which

involved four species *F. fujikuroi, F. proliferatum, F. verticillioides* and an unidentified species. The results showed that the strains isolated from rice were able to produce a wide range of mycotoxins on artificial growth media, which suggests that it can represent a risk for consumers of contaminated rice if they can also produce these compounds during infections. *F. proliferatum* and *F. verticillioides* produced unlike *F. fujikuroi,* high levels of fumonisins. *F. fujikuroi* was however the only species able to produce gibberellic acid.

PERSPECTIVES

During the last four years the natural occurrence of the *Fusarium* mycotoxins deoxynivalenol, nivalenol, zearalenone, T-2 toxin, HT-2 toxin, Fumonisins B1 and B2, moniliformin, beauvericin and enniatins has been examined in Danish maize at harvest in several studies, which all concluded that these mycotoxins are not the likely cause of health problems in dairy cattle herds (Nielsen et al., 2005a; Nielsen et al., 2006; Nielsen et al., 2007; Nielsen et al., 2008; Sørensen et al., 2007; Sørensen et al., 2008). If the problems in dairy herds are caused by *Fusarium* mycotoxins it will therefore be as a result of high levels of some of the mycotoxins which have not been examined. A potential source is *F. equiseti* which was found to be one of the most frequent species in maize (Sørensen et al., 2007) (**II**). This species produces several metabolites which have not been surveyed, including the type A trichothecenes scirpentriol (SCR), monoacetoxyscirpenol (MAS) and diacetoxyscirpenol (DAS) (Hestbjerg et al., 2002). In addition to *F. equiseti* one or more of these mycotoxins can also be produced by *F. poae, F. sporotrichioides, F. sambucinum* and *F. venenatum* (Thrane et al., 2004; Thrane and Hansen, 1995), which can also be found in Danish maize. The toxicity of SCR, MAS and DAS is reported to be similar to the other high potent type A trichothecenes T-2 and HT-2 toxin (Richardson and Hamilton, 1990; Thompson and Wannemacher, 1986). It may therefore be necessary to examine the occurrence of SCR, MAS and DAS to fully exclude *Fusarium* mycotoxins as the primary cause of the problems in the dairy cattle herds. In a study of the natural occurrence of 16 *Fusarium* mycotoxins in cereal grains and various maize products (including kernel, plants and silage samples), SCR, MAS and DAS levels were found to be comparable to T-2 and HT-2 levels (Schollenberger et al., 2006). In the four years where the occurrence of T-2 and HT-2 were surveyed in Danish maize, the level of 500 ppb, which is assumed to be the maximum level, which may be set by the commission of the European communities, was only exceeded in two samples. If the levels of SCR, MAS and DAS are similar to T-2 and HT-2 in Denmark it is unlikely that they can be responsible for the problems.

To exclude the possibility that the problems are caused by *Alternaria* mycotoxins it is necessary to examine the occurrence of some of the mycotoxins produced by *A. arborescens* and *A. tenuissima*, which are also found in Danish maize. The two species can produce a wide range of metabolites including tenuazonic acid, alternariols and altertoxins and as there is currently limited information on the natural occurrence of these compounds a survey of Danish maize is needed to fully exclude them as a cause of the problems in dairy herds. Surveys of *Alternaria* mycotoxins have shown that they can be present in high levels when conditions favor severe *Alternaria* infections (Azcarate et al., 2008; Li and Yoshizawa, 2000; Webley et al., 1997) and the long growth season of maize in Denmark may favor production of *Alternaria* mycotoxins.

If the source of the problems in the Danish dairy herds is mycotoxins produced by fungi infecting growing maize the cause may not be *Fusarium* or *Alternaria*, but some of the other genera which grow on maize. An example is the endophyte *Acremonium zeae*, which infects maize systematically without causing symptoms. This species is able to produce several antimicrobial compounds, which can protect maize plants against infections with *Aspergillus flavus* and *F. verticillioides* (Poling et al., 2008; Wicklow et al., 2005). Due to their microbial properties these compounds may also affect rumen microorganisms, although this has not been tested yet. Endophytes are unfortunately difficult to isolate because they usually grow slowly on artificial media and are therefore often out-grown by the other fungi present. It has therefore not been possible to isolate this species from Danish maize samples and it it still unknown whether it is present. However, as the

metabolites produced by *A. zeae* have not been suspected to be harmful to farm animals it they must be regarded as an unlikely candidate as the source of the problems.

As it seems unlikely that mycotoxins produced in the fields are causing the problems in dairy farms it is necessary to examine the occurrence of mycotoxin in maize silage produce by the fungi that are able to grow in the silage stacks. The most frequent mycotoxin producing species isolated fungi isolated from maize *Penicillium roqueforti* and the related *Pen. paneum* (Auerbach et al., 1998; O'Brien et al., 2005; Pelhate, 1977) and spores of *Pen. roqueforti* are almost always present in even healthy looking silage samples (Storm et al., 2008) (**I**). Other frequent mycotoxin producing contaminants of maize silage are *Monascus ruber* (Schneweis et al., 2001), *Aspergillus fumigatus* and *Byssochlamys nivea* (Nout et al., 1993; Richard et al., 2007). Two mycotoxins produced by *M. ruber*, Monacolin K_A and monacolin K_L, have been detected in high levels in maize silage with levels up to 15.6 and 65.4 ppm, respectively, which may be harmful to rumen microorganisms (Schneweis et al., 2001). Several mycotoxins produced by *Pen. roqueforti* and *P. paneum* have also been detected in high levels (Auerbach et al., 1998; O'Brien et al., 2006). The occurrence of fungi and some of their mycotoxins is currently being examined by project partners and their results will hopefully reveal if the mycotoxins produced in the silage stacks are a threat for dairy cattle.

Given that maize silage may contain a wide range of mycotoxins produced by various genera before or during the ensiling process there is a risk that some of the mycotoxins may have additive or even synergistic effects. There is however little knowledge on synergistic effects of the mycotoxins produced from different genera (Creppy, 2002). More studies on this subject are therefore needed to get the full picture of the impact of the mycotoxins present in maize silage

There is of course also the possibility that the problems were not caused by mycotoxins but resulted from some of the other changes in farming practice which occurred in the same period as the huge increase in the use of maize silage occurred. For instance, many farmers went from keeping their cattle fixed during winter to systems where cattle were free in this period. In addition the average herd size increased also in this period and all these changes may have had some influence on health problems in dairy cattle herds.

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APPENDIX 1 - PHYLOGENETIC ANALYSIS OF SELECTED *FUSARIUM* SPECIES

The Translation elongation factor 1α (TEF-1α) sequences of all target species were obtained from the *Fusarium*-ID v1.0 database (Geiser et al., 2004) (http://isolate.fusariumdb.org/index.php) except *F. sporotrichioides*, which was obtained from GenBank. Most of these sequences are also available in GenBank (**Table A1**).

Table A1. *Fusarium* TEF-1α sequences used for phylogenetic analysis.

Species	FUS. ID v1.0	Alt. ID	GenBank
F. avenaceum	FD_01317	NRRL 31101	
F. cerealis	FD_01107	NRRL 13393	AF212467.1
F. culmorum	FD_01111	NRRL 25475	AF212463.1
F. equiseti	FD_01321	NRRL 25795	
F. fujikuroi	FD_01169	NRRL 13566	AF160279.1
F. graminearum	FD_00985	NRRL 34079	AY452958.1
F. langsethiae	FD_00927	VI 01270	AJ420827.2
F. poae	FD_01308	NRRL 13714	
F. proliferatum	FD_01168	NRRL 22944	AF160280.1
F. sambucinum	FD_01307	NRRL 22203	AJ543602
F. sporotrichioides		VI01320	AJ420820.1
F. subglutinans	FD_01160	NRR_ 22016	AF160289
F. tricinctum	FD_01315	NRRL 25481	
F. venenatum	FD_01306	NRRL 22196	
F. verticillioides	FD_01185	NRRL 22172	AF160262.1

The obtained sequences were aligned in BioEdit (Hall, 1999) using the Clustal W (Thompson et al., 1994). The aligned sequences were then analyzed with Phylip 3.68 (Felsenstein, 2008) using dnapars to create the most parsimonious tree with 1000 bootstraps. The tree was finally visualized with TreeView (Page, 1996).

APPENDIX 2 - DETECTION OF *ALTERNARIA INFECTORIA* AND *PHOMA POMORUM* METABOLITES IN MAIZE

METABOLITE PRODUCTION ON ARTIFICIAL MAIZE MEDIUM

Selected strains of *A. infectoria* and *Ph. pomorum* were transferred with three point inoculation to a special made maize medium (100 g finely blended pieces from whole maize plants and 20 g agar per 1L medium). Metabolites were extracted after two weeks of incubation and analyzed with HPLC-UV-VIS, both as described elsewhere (Sørensen et al., 2009a) (**VI**).

DETECTION OF INFECTOPYRONES AND DIAPORTINIC ACID IN MAIZE WITH LC-MS/MS

Liquid chromatography was performed on an Agilent (Torrance, CA) 1100 HPLC system controlled by MassLynx V4.1. Extracts were separated on a Gemini C6-Phenyl 3 µm 2 mm i.d. × 50 mm column (Phenomenex) using a constant flow of a 0.3 mL/min MeCN/water gradient starting at 20% MeCN, which was increased linearly to 55% in 6 min and to 100% after two additional min. The column was washed with 100% MeCN for 2.5 min at 0.5 mL/min before reverting to the 20% MeCN in 2 min, maintaining this for 5 min. The water contained 20 mM ammonium formate. The LC was coupled to a triplequadrupole mass spectrometer (Waters-Micromass, Manchester, U.K.) with Z-spray ESI operated in positive mode source using a flow of 700 L/h nitrogen at 350 °C; hexapole 1 was held at 50 V. The system was controlled from MassLynx 4.1 (Waters-Micromass). Nitrogen was also used as collision gas, and the MS operated in MRM mode (dwell time = 200 ms) with the parameters shown in **Table A2**.

Table A2. MS/MS Method Including Scan Events, Retention Time (RT), Transition Ions, and the Cone and Collision Energies (CE) used.

	RT	Ion type	Transition (*m/z*) [a]	Cone (V)	CE (V)
Diaportinic acid	5.3	Quantifier	281.1 → 235.0	20	15
		Qualifier	281.1 → 190.9	20	30
Infectopyrones	5.9	Quantifier	265.0 → 203.0	25	15
		Qualifier	265.0 → 139.0	25	30

[a] Both transitions were made from $[M+H]^+$

APPENDIX 3 - METABOLITE ANALYSIS OF THE *GIBBERELLA FUJIKUROI* SPECIES COMPLEX

Metabolites were extracted and gibberellic acid and fumonisin B1 were analyzed as described elsewhere (Wulff et al., 2009). Reference standards of moniliformin (MON), fusarin A and C (FA and FC), fusaproliferin (FUP) and beauvericin (BEA), which were available from previous studies (Nielsen and Smedsgaard, 2003), were used to identify these metabolites in the fungal extracts. MON and FUP were detected using HPLC-UV-VIS using the same chromatography as previously described (Sørensen et al., 2009a) (**VI**). FA, FC, FUP and BEA were detected by LC-MS using the same chromatographic conditions as previously described (Wulff et al., 2009). The $[M+H]^+$ ions with mass ± 0.02 amu were used to detected the metabolites.

ORIGINAL MANUSCRIPTS (I-VIII)

I. Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart Postharvest Review* **4:** 6.

II. Sørensen, J.L., Nielsen, K.F., and Thrane, U. (2007) Analysis of moniliformin in maize plants using hydrophilic interaction chromatography. *Journal of Agricultural and Food Chemistry* **55:** 9764-9768.

III. Sørensen, J.L., Nielsen, K.F., Rasmussen, P.H., and Thrane, U. (2008) Development of a LC-MS/MS method for analysis of enniatins and beauvericin in whole fresh and ensiled maize. *Journal of Agricultural and Food Chemistry* **56:** 10439-10443.

IV. Sørensen, J.L., Mogensen, J.M., Thrane, U., and Andersen, B. (2009) Potato carrot agar with manganese as an isolation medium for *Alternaria*, *Epicoccum* and *Phoma*. *International Journal of Food Microbiology* **130:** 22-26.

V. Andersen, B., Sørensen, J.L., Nielsen, K.F., van den Ende, A.H.G. and de Hoog, S. (2009) A polyphasic approach to the taxonomy of the *Alternaria infectoria* species-group. *Fungal Genetics and Biology* **46**: 642-656.

VI. Sørensen, J.L., Aveskamp, M.M., Thrane, U., and Andersen, B. (2009) Polyphasic characterization of *Phoma pomorum* isolated from Danish maize. (Submitted to *International Journal of Food Microbiology* 1st May 2009).

VII. Sørensen, J.L., Phipps, R.K., Nielsen, K.F., Frank, J., Schroers, H.J., and Thrane, U. (2009) Analysis of *Fusarium avenaceum* metabolites produced during wet apple core rot. *Journal of Agricultural and Food Chemistry* **57:** 1632-1639.

VIII. Wulff, E., Sørensen, J.L., Lübeck, M., Nielsen, K.F., Thrane, U., and Torp, J. (2009) Genetic diversity, extrolite production and pathogenicity of *Gibberella fujikuroi* species complex associated with rice seeds originating from Africa and Asia. *Environmental Microbiology and Environmental Microbiology Reports* (Accepted with minor revisions 30th June 2009).

ORIGINAL MANUSCRIPT (I)

Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart Postharvest Review* **4:** 6.

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Mycotoxins in silage

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Abstract
Purpose of review: This paper reviews the present knowledge on mycotoxins in silage, focusing on grass and maize silage. This includes the occurrence of filamentous fungi pre- and postharvest, possible and confirmed mycotoxins in silage, toxicological concerns and means to prevent the problem.
Findings: Preharvest contamination of grass and maize by *Fusarium*, *Aspergillus* and *Alternaria* can lead to contamination of silage. Well known mycotoxins deoxynivalenol (DON), zearalenone (ZEA), fumonisins and aflatoxins have been detected in silages but concentrations seldom exceed regulatory limits. It also appears that DON, ZEA and fumonisins are degraded in silage, but exact mechanisms are unknown. Postharvest spoilage is dominated by *Penicillium roqueforti*, *Aspergillus fumigatus* and Zygomycetes. Both *P. roqueforti* and *Asp. fumigatus* produce a wide range of secondary metabolites, some of them confirmed mycotoxins, others with antimicrobial or immunosuppressive effects. Some fungal metabolites have been detected in silage but many have not been looked for. Evidence for acute toxicosis caused by contaminated silage is rare. Mycotoxins in silage are more often associated with less specific symptoms like ill-thrift or decreasing yield. This may be caused by long-term exposure to the complex mixture of secondary metabolites that silage can contain. Mycotoxins with antimicrobial effects may also affect ruminant digestion. To prevent postharvest spoilage of silage the most important factor is omission of oxygen. Additives can improve certain silage properties but they are not conclusively an advantage and cannot replace good silage management.
Directions for future research: The effects of long-term exposure and of complex mixtures of bioactive fungal compounds are subjects of interest. Especially high-yielding livestock may be subject to sub-acute symptoms under these conditions. There is also a need for analytical methods with specificity and accuracy to determine many of the less known mycotoxins and secondary metabolites in silage as well as possible unknown compounds.

Keywords: silage; grass; maize; mycotoxins; preharvest; postharvest

Abbreviations

DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
ELEM	Equine Leukoencephalomalacia
FB1	Fumonisin B_1
LC-MS	Liquid Chromatography–Mass Spectrometry
NIV	Nivalenol
PPE	Porcine Pulmonary Oedema Syndrome
ZEA	Zearalenone
ZOL	Zearalenol

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Introduction

Ensiling is used worldwide as a simple and effective way to preserve forage for livestock, using a natural lactic acid fermentation of the feedstuffs which is acidified and can subsequently be stored for long periods without degrading. In modern agriculture, large amounts of silage are consumed each day all year round with dairy cows eating up to 40 kg/day. In Western Europe the total area of land harvested for silage has remained stable at around 15 million hectares since 1990 and worldwide the silage production has tended to increase from 1989–2000 [1]. The use of especially maize silage has increased over the last few decades with the availability of short season maize varieties suitable for temperate climates [1, 2]. In Denmark the production has increased by 500% from 1990 to 2007 [3] and maize silage is very widely used for cattle in both dairy and meat production.

The most common silage crops are grass and maize, but many other products like whole-crop barley, alfalfa, clover, sugar-beet tops, and residues from sugar production can be preserved as silage [4**]. A thorough review of silage making from crops to nutritive value is covered by McDonald *et al.* [4**]. In all cases the product is harvested, cut in suitable sizes and packed tightly in either silos, stacks or bales, and sealed to avoid oxygen infiltration. Residual enzymatic activity of the plant and microbial respiration of the carbohydrates released by chopping quickly depletes the small amount of O_2 in the stack and raises the concentration of CO_2. One hour after ensilage, O_2 levels in the range from 1 to 2% and CO_2 from 20 to 90% were recorded in baled silage [5]. This selects for the proliferation of natural lactic acid bacteria, whose numbers increase from below 10^2–10^5 CFU/g on plants in the field to 10^9–10^{10} CFU/g in silage that is only a few days old [4**, 6]. They ferment sugars to primarily lactic acid and acetic acid, lowering pH to ~4 or less. Clamp and baled grass silages have been reported to have an average pH of 4.0 and 4.8, respectively [5]. The combination of low O_2 concentration, high CO_2 concentration and a low pH makes silage a very hostile environment for spoilage organisms including bacteria, yeasts and filamentous fungi.

Nevertheless, growth of filamentous fungi is frequently observed in silage. This constitutes a loss of nutritive value for the farmer and, much worse, a risk for contamination with mycotoxins. Toxins in the feed may constitute a health risk for animals and there is also the risk for carry-over to humans via milk and meat [7*]. In the last 30 years, cases of ill-thrift, disease and death in livestock have been related to the presence of mycotoxins in silage [8*–12] and the issue is much debated [2, 7*, 13–18**]. Infestation and subsequent mycotoxin production may take place both pre- and postharvest and silage can thus be contaminated with both well known *Fusarium* toxins like deoxynivalenol (DON) and zearalenone (ZEA), as well as less known secondary metabolites from species of *Penicillium* and other fungi.

This article reviews the present knowledge on mycotoxins in silage including pre- and postharvest contaminants, toxicological issues and means for preventing the problem. The focus is on grass and maize silage for cattle as these are considered the economically most important use of silage crops.

Toxigenic field fungi

The three most important toxigenic genera occurring preharvest in cereals and maize are *Aspergillus*, *Fusarium* and *Alternaria* (Table 1). *Alternaria* and *Fusarium* are often categorised as field fungi whereas some species of *Aspergillus* can occur both pre- and postharvest. The occurrence of these fungi is influenced by several factors, including agricultural practices (crop rotation, crop variety, fertilisation and cultivation methods) and climatic conditions (temperature and moisture).

Small-spored *Alternaria* are common pathogens of small grains and maize with *Alt. alternata, Alt. arborescens, Alt. infectoria* and *Alt. tenuissima* as the predominant species [19]. *Alt. alternata* may not be as common as the literature indicates, as it is often mis-identified. Of these species *Alt. infectoria* is the only one with a known sexual stage (*Lewia*). The infections often occur in the late growth season as black spots on the host plants.

The two predominant toxigenic field *Aspergillus* species are *Asp. flavus* and *Asp. parasiticus*. These two species are mainly found in warm arid, semi-arid and tropical regions and cause huge problems in the Midwestern corn belt in the USA [20]. They can infect growing maize and produce mycotoxins preharvest but may apparently also survive the ensiling process, as findings of *Asp. flavus* in silages have been reported [21–23*].

Species of the anamorphic genus *Fusarium* are destructive pathogens responsible for several diseases including red/pink ear rot of maize and head blights of wheat. In areas with temperate climate, *F. avenaceum, F. culmorum* and *F. graminearum* (teleomorph: *Gibberella zeae*) are the predominant species, whereas the members of the *Liseola* section *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (teleomorph: *Gib. moniliformis*) dominate in warmer parts of the world [24].

Several additional producers of bioactive secondary metabolites are often associated with cereal and maize including species of *Epicoccum, Cladosporium, Diplodia* and *Phoma*. The natural occurrence of mycotoxins produced by these genera in food and feeds has not been studied yet and an estimation of their importance is therefore not possible. *Diplodia* toxins have however been suggested as the primary cause in an Argentinean case where 10 heifers died from eating mouldy maize infected with *Diplodia maydis* [25]. Attention should therefore be given in the future to mycotoxins produced by other genera than *Aspergillus* and *Fusarium*.

Several species of the sexual genus *Epichloë* (anamorph: *Neotyphodium*) are endophytes of some varieties of pooid

Table 1. The most common species of *Fusarium, Alternaria* and *Aspergillus* in preharvest silage crops, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage
Fusarium culmorum, *F. cerealis* and	Culmorin	
F. graminearum	Deoxynivalenol	+
	3- or 15-Acetyl deoxynivalenol	+
	Nivalenol	+
	Fusarenone-X	
	Fusarins	
	Zearalenones	+
	2-Acetylquinazolinone	
	Aurofusarin, Rubrofusarin, Butenolide, Chrysogine	
F. proliferatum, F. subglutinans and	Beauvericin	+
F. verticillioides	Fumonisins	+
	Fusaproliferin (*F. pro*. and *F. sub*.)	
	Fusapyrone (*F. pro*), Fusaric acid	
	Moniliformin (*F. pro.* and *F. sub.*)	
	Naphthoquinone pigments	
F. poae and *F. sporotrichioides*	Aurofusarin	
	Beauvericin	+
	Chrysogine (*F. sporotrichioides*)	
	Culmorin	
	Scirpentriol	+
	Monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol	
	Enniatins	+
	Fusarenone-X (*F. poae*)	
	T-2 toxin	
	HT-2 toxin	+
	Neosolaniol	
	Nivalenol (*F. poae*)	+
F. avenaceum and *F. tricinctum*	2-Amino-14,16-dimethyloctadecan-3-ol (*F. ave*.)	
	Acuminatopyrone (*F. ave*.), Antibiotic Y, Aurofusarin	
	Beauvericin	+
	Butenolide	
	Chlamydosporols	
	Chrysogine	
	Enniatins	+
	Fusarins, Gibepyrone A, Moniliformin, Visoltricin (*F. tric*)	
F. equiseti	Nivalenol	+
	Scirpentriol, monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol, Equisetin, Fusarenone-X	
	Fusarochromanone, Chrysogine	
Alternaria alternata, Alt. arborescens	AAL-toxins (*Alt. arborescens*)	+[a]
and *Alt. tenuissima*	Alternariols, Altertoxins, Tentoxin, Tenuazonic acid	
Alternaria infectoria	Infectopyrones, Novae-zelandins	
Aspergillus flavus and Asp. parasiticus	Aflatoxin B_1 and B_2	+
	Aflatoxin G_1 and G_2 (*A. parasiticus*)	
	Aspergillic acid	
	Cyclopiazonic Acid	+
	Kojic acid	
	Sterigmatocystins	+
	Versicolorin and precursors, 3-Nitropropionic acid	
	Aflavinine, Aflatrem	

[a]Needs reconfirmation

Figure 1. Ball of maize silage infected with *Penicillium roqueforti*, which was observed in the middle of a well managed silage stack.

grasses [26]. *Epichloë* species can infect plants through wounds or stigmata and by seed-transmission whereas the asexual *Neotyphodium* species are only seed-transmitted. The endophytes colonise host plants systematically without causing disease symptoms. Several bioactive alkaloids can be produced by the endophytes during the infections, which are beneficial to the host plant as they can be active against feeding insects or herbivores. Other fitness improvements that may be attributed to the symbiosis between host plants and endophytes include growth stimulation and enhanced drought tolerance [27]. Some neurotoxic alkaloids have been implicated in livestock toxicosis, including ergot alkaloids [28] and lolitrems [29*]. For reviews on this subject see [16, 18**] The recognised toxicosis events occurred when livestock had been feeding on *Epichloë* or *Neotyphodium* infected grass, but so far nothing is known about their occurrence and stability in grass silage.

Postharvest contamination

The ensiling process eliminates most fungi from the field [30*, 31]. There are however other species of filamentous fungi that are able to tolerate both organic acids, carbon dioxide and the low availability of oxygen (Table 2).

The most commonly found filamentous fungi in silage are *Penicillium roqueforti* and the closely related *P. paneum* [22, 32*, 33–35]. *P. roqueforti* has its pH optimum between pH 4 and 5 [36], tolerates high levels of CO_2 [37] as well as the different organic acids commonly found in silage [22, 38]. The optimum temperature is 25°C but *P. roqueforti* may grow at 5°C [38]. Thus it is able to grow in silage all year round, even in temperate climates. *P. roqueforti* also sporulates heavily and spores are almost always present even in healthy looking maize silage [Storm IMLD, unpublished]. Growth of *P. roqueforti* and *P. paneum* is often seen in silage either in layers, on the surface or as lumps as big as 40 cm in diameter in the middle of stacks (Figure 1). The colour is green often in grey or blue shades and *P. roqueforti* and *P. paneum* cannot be differentiated visually on the silage.

Other very common fungi are various species of *Mucor* and *Rhizopus* (class Zygomycetes), which have been isolated from all types of silage [21–23*, 31, 32*–34, 40*]. They grow rapidly especially in partly aerated outer layers of silage. The rapid growth of these species may obscure the growth of other less vigorous species during cultivation and identification in the laboratory.

Aspergillus fumigatus has also been isolated from silages all over the world, both in warm [21, 41] and temperate [22, 23*, 33, 34, 40*] climates. It has a high temperature optimum and tolerates temperatures up to 55°C [42] and can therefore often be observed near degraded outer layers of silage stacks where the microbial heat from degradation has selected for heat-tolerant species.

Other species often encountered are *Monascus ruber* [23*, 33, 40*, 43] and *Byssochlamys nivea* [22, 23*, 44]. *M. ruber* often produces red pigments and can be seen as lumps both near surfaces and in central parts of silage stacks. *B. nivea* and the anamorphic form *Paecilomyces niveus* produce white colonies in silage. *B. nivea* can survive acidic and anaerobic conditions and the ascospores are heat-resistant, as illustrated by the fact that it is an important contaminant of canned fruit and fruit juices [42].

Fusarium spp. have been isolated from silage in several cases [21, 23*, 40*]. Fusaria are generally not capable of surviving the ensiling process. Only *F. oxysporum* is known to survive under acidic and anoxic conditions [42]. Mansfield and Kuldau [30*] registered several species of *Fusarium* in fresh maize but none after ensiling. The survival of spores or recolonisation after opening may explain findings of Fusaria in silage.

Classic mycological determination of mycobiota by dilution and plating may unfortunately not reflect the actual growth of filamentous fungi in field and silage. This is a classic mycological dilemma already mentioned in a review of silage mycology by Pelhate [31]. The use of suitable media and incubation in modified atmosphere may give a more representative picture of the actual mycobiota in silage, but standardised procedures need to be developed. Even so heavily sporulating species like *P. roqueforti* may be overestimated. Silage cannot be considered a homogenous medium either. Within a stack or bale there are many ecological niches. For instance *P. roqueforti* is often observed as layers at a depth of 20–80 cm [Storm IMLD, unpublished, 33] where the O_2 concentration is too low for most spoilage organisms. In the outer layers *P. roqueforti* has been out competed by yeasts, bacteria and other filamentous fungi. Molecular biological techniques can in theory reveal the presence of all fungi in

Table 2. The most common fungal postharvest contaminants of silage, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage[a]	Reference
Penicillium roqueforti[b]	Agroclavine	+	[8]
	Eremofortin C		
	Mycophenolic acid	+, 1.3, 35, 117	[2, 8, 34, Nielsen KF, unpublished]
	PR-toxin	+	[Nielsen KF, unpublished]
	PR-amide and PR-imine		
P. roqueforti	Roquefortine A, D, 16-OH-roquefortine	+	[8]
and *P. paneum*[b]	Roquefortine C	+, 5.7, 36, 50	[8, 35, 82, Nielsen KF, unpublished]
	Andrastin A, B and C	+	[8, Nielsen KF, unpublished]
	Citreoisocoumarin	+	[8]
	Orsellinic acid		
	Festuclavine	+	[8]
P. paneum[b]	Marcfortine A	+	[8]
	Marcfortine B and C		
	Patulin	1.2, 40	[44, 82]
	Gentisic acid		
Aspergillus fumigatus[c]	Gliotoxin	0.878	[23*]
	bis-dethio-bis(methylthio)-gliotoxin	+	[Nielsen KF, unpublished]
	Fumigatins		
	Trypacidins		
	Sphingofungins		
	Pseurotins		
	Helvolic Acid		
	Fumagillins		
	Fumigaclavines		
	Fumitremorgines		
	Diketopioperazines		
	Fumiquinazolines		
Byssochlamys nivea/	Patulin	1.2, 40	[44, 82]
Paecilomyces niveus	Byssochlamic acid		
	Mycophenolic acid	+, 1.3, 35, 117	[8, 34, 82, Nielsen KF, unpublished]
Monascus ruber	Citrinin	0.037, 0.064, 0.25	[23*, 40*, 43]
	Monacolins	65	[43]
	Pigments, eg, ankaflavin		
	Monascopyridines		
Zygomycetes	May cause zygomycosis especially in immunocompromised animals		[76]
Geotrichum candidum	May reduce palatability of silage		[31]

[a]+: Metabolite detected in silage samples. Numbers state maximum concentrations in mg/kg where quantitative determination has been performed.
[b]Based on [8, 103*]
[c]226 Extrolites registered by Frisvad *et al.* [75*]

silage. Mansfield and Kuldau [30*] compared a DNA-sequence based technique with plating on malt-yeast sucrose agar (MYSA) and Nash medium (NASH) and found a much greater abundance of species with the molecular technique. Again dormant spores can give misleading results and the quantity of DNA cannot be correlated with the amount of mycotoxins.

Mycotoxins and other secondary metabolites

The above mentioned fungi are known to produce a wide range of mycotoxins and other secondary metabolites. But the production of these is very substrate dependent and not all may be present in silage. The complex microbial ecosystem of silage can also account for degradation and binding of such compounds.

Preharvest

Of the *Fusarium* derived mycotoxins, the trichothecenes are sesquiterpenes and are produced by various species of *Fusarium*. The compounds are divided into type A and B trichothecenes. Type A trichothecenes (mainly diacetoxyscirpenol [DAS], T-2 toxin and deacetylated analogues of these) are mainly produced by *F. poae*, *F. sporotrichioides* and *F. langsethiae* and are considered more toxic than type B trichothecenes (mainly DON and nivalenol [NIV], fusarenone-X, 3- and 15-acetyl-DON as well as acetylated and deacetylated analogues of these), which are primarily produced by *F. cerealis*, *F. culmorum* and *F. graminearum* [45]. Trichothecenes have a variety of toxic effects like vomiting (DON), reduced feed uptake and immuno-suppression as the most pronounced [46]. DON is usually the predominant trichothecene in crops and is therefore also the best studied. ZEA and α- and β-zearalenol (α- and β-ZOL) are estrogenic compounds mainly produced by the trichothecene type B producing *Fusarium* species [45]. In a survey of mycotoxins in various Dutch silage types, DON and ZEA were almost completely absent in grass silage, while they were highly abundant in maize silage [47*], despite the absence of the producing organisms postharvest [30*].

Fumonisins are sphinganine analogues with carcinogenic properties [48] and are primarily produced by *F. proliferatum* and *F. verticillioides* [45]. These species are mainly present in tropical and subtropical areas and fumonisin contaminations of preharvest crops are therefore higher in these areas. There are several groups of fumonisins with several members, but fumonisin B_1 (FB1) is the predominant and best studied analogue.

DON and FB_1 were shown to be less stable than ZEA in a lab scale experiment with ensiled maize [49*]. The maximum toxin degradation observed for DON, FB_1 and ZEA was 100%, 92% and 53%, respectively [49*]. The experiments also showed that storage time and dry matter content are more important than temperature. In a study of fresh and ensiled maize, DON levels were reduced by 57% in 3–6 month old silage stacks [50]. These observations suggest a substantial degradation of DON during ensiling, which is a fate that the other trichothecenes are likely to share. Some removal of field produced mycotoxins can be attributed to lactic acid bacteria. *In vitro* studies suggest that binding of DON, ZEA and FB1 is the major mode of action for lactic acid bacteria [51].

Plants are able to reduce the toxicity of mycotoxins formed in the fields for example by conjugation of mycotoxins to polar substances such as sugars, amino acids or sulphate. Natural occurring glucoside conjugates of ZEA [52] and deoxynivenol [53] have been detected. The conjugated forms will not be detected by standard methods designed for the precursor mycotoxins as they may be harder to extract and have altered chromatography. This means that the actual amount of mycotoxins may be underestimated due to masked conjugated mycotoxins.

Species of *Fusarium* can produce several other types of mycotoxins in cereals and maize preharvest, including moniliformin, fusaproliferin, beauvericin and enniatins, but very little is known about their stability in silage. The predominant enniatin analogue, enniatin B, was detected at levels up to 218 ng/g in 3-month-old maize silage stacks, while the related beauvericin occurred less frequently and at levels up to 63 ng/g. Enniatin levels in 3, 7 and 11 month old silage were not different from each other but were all lower than in freshly harvested maize [54]. This suggests that some of the enniatins were degraded within the first 3 months. In another study of preharvest maize, moniliformin was only produced in insignificant low ppb levels [55].

The four most frequently occurring *Alternaria* species in cereals and maize are *Alt. arborescens, Alt. alternata, Alt. tenuissima* and *Alt. infectoria,* which are able to produce a wide range of compounds with disputed toxicity. *Alt. arborescens, Alt. alternata* and *Alt. tenuissima* can produce alternariols, altertoxins, altenuene and tenuazonic acid [56], but there are only few reports on the natural occurrence of these compounds in small grain cereals preharvest, summarised in [57]. *Alt. infectoria* can produce infectopyrones and novaezelandins [58], but their natural occurrence has not been studied. One paper [59] also reports finding the *Alternaria* mycotoxins AAL-toxin A and B in silage. Liquid chromatography–mass spectrometry (LC-MS) with only one SIM ion (not very specific in such dirty matrix) was used to substantiate this very interesting finding, and since only one isolate (tomato pathogen *Alt. arborescens,* syn. *Alt. alternata* f. sp. *lycopersici*) in the world until now has been found to produce AAL toxins, the findings of AAL toxins in silages seems unlikely and needs proper validation.

With *Aspergillus flavus* and *Asp. parasiticus* present in crops and silage, aflatoxins may be produced. These are the most important group of mycotoxin produced by this organism, and mainly includes the B_1, B_2, G_1 and G_2 analogues, which are all produced by *Asp. parasiticus*, whereas *Asp. flavus* can

only produce B_1 and B_2 [60]. Aflatoxins are the most carcinogenic of known secondary metabolites and their occurrence in silage can be of great concern to human health as they can be transformed by cattle to hydroxylated derivates (aflatoxins M_1 and M_2), which can be found in meat and milk products. Other mycotoxins from *A. flavus* are cyclopiazonic acid and 3-nitropropionic acids. Aflatoxin B_1 has been detected in silage in some surveys while others have looked for it with negative results (Table 3)

Postharvest

P. roqueforti and *P. paneum* are the most widespread species of filamentous fungi in silages and they have on several occasions been associated with ill-thrift and disease in cattle herds [8, 10, 11]. As seen in Table 2 they produce a wide range of secondary metabolites *in vitro* and many of them have also been detected in silage.

The roquefortines are very ubiquitous and have therefore been suspected to be involved in toxicoses [61]. Data on neurotoxicity [62] and antibiotic properties [63] are published but no acute toxicity and a low transfer to organs and tissue were observed in feeding experiments with sheep [64]. PR-toxin (only produced by *P. roqueforti*) on the other hand has acute toxic effects in rats and mice [62, 65] but its fate in ruminants is unknown. Another known toxin, patulin, is produced by *P. paneum* as well as *B. nivea*. Patulin damages the kidneys and the gastro-intestinal tract functions in rats [66] and may reduce male fertility [67]. It has antibiotic properties [68] and is immunosuppressive at high doses [69, 70]. It does however form adducts with S-containing amino acids [71, 72] and may therefore not be bio-available in ruminants. Another commonly encountered metabolite is mycophenolic acid, which is produced by both *P. roqueforti* and *B. nivea*. It is antibiotic and immunosuppressant [73, 74]. The andrastins and marcfortines have not been tested in higher animals. The clavines are similar to alkaloids produced by *Neotyphodium* endophytes in Fescue grass preharvest and may thus result in similar symptoms, however ergovaline is considered the most important toxin involved in Fescue toxicosis. In a recent survey by Driehuis *et al.* [47*] roquefortine C was reported only in 1 of 120 grass silages and none of 140 maize silages. Mycophenolic acid was not found in any samples. Sampling of the silages was however conducted only 1–2 months after harvest and the stacks were still completely sealed so growth of postharvest contaminants was unlikely.

The widespread presence of *Asp. fumigatus* in silage naturally calls for concern. It is a known producer of more than 200 secondary metabolites [75*], including the potent gliotoxin, and may cause invasive infections in animals (Aspergillosis) [76]. Many of the metabolites are known to have antimicrobial, antifungal or antiprotozoan effects [75*] and may thus affect the microbiota of the rumen. Others, like gliotoxin, are immunosuppressive [77]. Silage samples contaminated with *Asp. fumigatus* have been analysed for gliotoxin only, which is produced in highest amount on substrates with a low C/N ratio. Gliotoxin may therefore not be a very good marker for presence of *Asp. fumigatus* toxins in silage. In *Monascus ruber* infected silage, citrinin has been detected. Citrinin is nephrotoxic [78], while the monacolins produced by the same species have no toxic effects and are used as cholesterol-lowering drugs.

Some Zygomycetes can, via endophytic bacteria, produce several bioactive secondary metabolites [79, 80], but the distribution of toxigenic isolates is not well examined. The fast growth of Zygomycetes may spoil large amount of silage very rapidly. Furthermore some species are known to cause invasive infections, Zygomycosis [76], especially in immuno-compromised individuals.

Toxicology

Mycotoxins in silage can affect animal health and productivity [18**]. Exposure of humans via transfer of mycotoxins to food (eg, milk) is also of concern [2, 18**]. The mycotoxins contaminating silage can induce carcinogenic, estrogenic or immunosuppressive effects. Feed refusal, birth defects, kidney, liver or lung damages, etc have also been observed in clinical trials [17], but acute intoxications causing death are rare [81]. Animals feeding on silage may be exposed to a mixture of mycotoxins [23*, 40*, 47*, 82] and chronic exposure to low levels of mycotoxins may result in non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances [18**, 83]. The intoxication of animals under field conditions does not always match the concentration of specific toxins [18**]. A cocktail of toxins can give a stronger effect than the single toxins alone [78]. Furthermore, not all toxins in silage are described in literature since new secondary fungal metabolites are still discovered [84, 85].

A review of animal disease outbreaks due to *Fusarium* toxin contaminated feed has been given by Morgavi and Riley [83]. Clear signs of exposure to a specific toxin are rare under field conditions; for DON feed refusal has been reported in cattle, pigs and chickens. Fumonisins can induce brain lesions in horses - equine leucoencephalomalacia (ELEM) and lung damage in pigs - porcine pulmonary oedema syndrome (PPE) [83]. Mouldy maize silage infected with *P. roqueforti* produced loss of appetite, disturbance of rumen activity and gut inflammation in dairy cows [86]. Kristensen *et al.* [87] however did not see any significant effects on milk yield or rumen pH in a feeding experiment where cows were fed alternating rations, including a ration with DON-contaminated maize silage and one with *Penicillium* contaminated maize silage. There were a few changes in the ruminal fermentation pattern that were significant.

Ruminants are often less susceptible to intoxication than other animal species. For instance they show lower responsiveness to DON, ZEA and fumonisins than pigs do [88–90]. The rumen microbiota can inactivate and degrade some mycotoxins, but not all types. For example, ochratoxin A is extensively degraded to the less toxic ochratoxin α [91], whereas ZEA is metabolised to the even more potent α -ZOL

Table 3. Confirmed examples of maize silage contaminated with *Fusarium, Aspergillus* and *Alternaria* toxins.

Mycotoxin	Country	Concentration (µg/kg)[a]		Reference
		Mean	Range	
Deoxynivalenol	Argentina		30–870	[104]
	France	160		[23*]
	France	204		[23*]
	Germany	2,919	?–3,944	[105]
	The Netherlands	651	nd–3,142	[47*]
	USA	600	nd–3,700	[50]
15-Acetyldeoxynivalenol	Germany	59	?–127	[105]
	The Netherlands	45	nd–1,013	[47*]
Nivalenol	Germany	1,612	?–2,809	[105]
HT-2 toxin	Germany	18	?–26	[105]
Scirpentriol	Germany	25	nd–124	[105]
Monoacetylscirpentriol	Germany	20	nd–49	[105]
Zearalenone	Argentina		nd–350	[104]
	France		<20	[23*]
	Germany	432	?–1,790	[105]
	The Netherlands	92	nd–943	[47*]
α-Zearalenol	Germany	3	nd–15	[105]
β-Zearalenol	Germany	23	nd–116	[105]
Fumonisin B_1	Argentina		340–2,490	[104]
	The Netherlands	463	nd–26,200	[47*]
	USA	2,020	nd–10,100	[59]
	USA	590	nd–1,824	[106]
Fumonisin B_2	The Netherlands	130	nd–7,800	[47*]
	USA	980	nd–20,300	[59]
	USA	66	nd–276	[106]
Fumonisin B_3	USA	29	nd –161	[106]
Enniatin B	Denmark	73	nd–218	[54]
Enniatin B_1	Denmark	10	nd–48	[54]
Beauvericin	Denmark	8	nd–63	[54]
Aflatoxin B_1	Argentina		nd–176	[104]
	Italy		nd–<4	[107]
	Mexico		500–5,000	[108]
	Brazil	nd		[109]
	USA	nd		[110]

[a]nd: not detected.

[89]. FB1 largely passes the forestomach in ruminants [90]. Animals with impaired rumen fermentation are expected to metabolise toxins less effectively. Patulin is an example of a mycotoxin with antibacterial properties that can disturb the rumen fermentation [92]. Keese *et al.* [93] have also detected alterations in the ruminal fermentation pattern when cows were fed a ration containing 5.3 mg/kg DM of DON. High-yielding dairy cows may be more susceptible to diseases caused by mycotoxins, maybe due to a higher level of stress [94].

Milk can be contaminated with the carcinogenic metabolite aflatoxin M_1 [95], when lactating animals are exposed to the mycotoxin aflatoxin B_1 in feedstuffs. Up to 6% of the administered dose of aflatoxin is excreted in the milk [96]. Carry over rates of DON, ZEA, ochratoxin A, and fumonisins from feed to milk are much lower than aflatoxin. Hence humans are not significantly exposed to these four toxins through milk [88–91]. The carry-over rates from feed to milk of *P. paneum* and *P. roqueforti* toxins, eg, PR-toxin, roquefortines or festuclavine are not known [2].

Many countries have regulatory limits for mycotoxins in feed. Maximum acceptable levels of DON (0.9–12 mg/kg feed), ZEA (0.1–3 mg/kg), ochratoxin A (0.05– 0.25 mg/kg) and fumonisins (5–60 mg/kg) in feed material have been set by the European Union. These values are toxin, feed-type, and animal dependent, and address animal welfare, as the exposure of humans through animal products is low [97]. Maximum levels of aflatoxin B_1 (0.005–0.02 mg/kg) in feed is regulated based on human safety as it is a genotoxic carcinogen [98]. The lowest value in feed applies to dairy cattle due to carry-over in milk. As seen in Table 3 mycotoxin levels in silage rarely exceed the existing regulatory limits.

Preventive agricultural practices

In order to minimise the risk of fungal spoilage and mycotoxin contamination of silage, farmers can implement different strategic and practical approaches.

Preharvest infection of crops cannot be eliminated. Incidents and concentrations of preharvest toxins are very dependent on weather conditions, and models to predict the spread of plant pathogens have been developed [99]. In a survey by Mansfield *et al.* [50] agronomic practices had no effect on incidence of DON, but the concentrations were significantly higher in no till-systems than in mixed till and mouldboard till systems.

To avoid spoilage of silage in silos and bales there are several practical approaches to consider. Proper chopping, thorough compaction and sealing are very important factors for limiting the oxygen supply, which is of utmost importance. O'Brien *et al.* [32*] found that visible damage to the polythene film of baled grass silage was the only bale production and storage characteristic that significantly predisposed bales to increased fungal spoilage. Furthermore, a positive correlation was observed between polythene film damage and dry-matter content [100] most likely because dry and stiff stems are more likely to puncture the film. For silage in stacks and silos, the compaction is very important both for the quick achievement of anaerobic conditions and for minimisation of O_2 infiltration from the cutting front. Therefore particle size must not be too big as this hinders compaction. Special equipment for cutting silage rather than grabbing it from the stack may also minimise O_2 infiltration. Proportionating silage stacks to the rate of use may also help, as low rate of use has been associated with spoiled silage [33]. Optimal dry-matter content of the crop is also important for the initiation and course of the silage fermentation. Significant negative correlation between dry matter content and concentration of lactic, acetic, propionic and butyric acid was observed [32*].

In order to affect the fermentation process, silage additives can be added during silage making. These may be acids intended to restrict growth of undesirable organisms from the start, fermentable sugars (eg, molasses) to stimulate production of organic acids or biological inoculants to increase the concentration of desired microorganisms in silage. Biological additives are the most popular type worldwide but may be used in combination with the other types [1]. Biological inoculants are however not always successful and there are both advantages and disadvantages to them [101].

Conclusion

Silage can contain a wide range of mycotoxins and other secondary metabolites originating from preharvest infection of crops or from postharvest infection in silos, stacks and bales. This has been associated with ill-thrift and disease in cattle, but the evidence for acute intoxication caused by contaminated silage is rare. Many of the filamentous fungi associated with silage are however producers of antimicrobial and immunosuppressive compounds. It is possible that complex mixtures of these may result in sub-acute symptoms, ie, impaired rumen function or increased susceptibility to infections. This subject calls for further investigation.

The mycobiota of silage has been examined in several cases around the world, and the results are fairly consistent with *P. roqueforti* and *Asp. fumigatus* as some of the most abundant species. An often encountered group of filamentous fungi is the Zygomycetes but the possible effects of these have not been examined. The interplay between filamentous fungi, bacteria and yeasts is also an issue of interest, which may be able to explain the occurrence of filamentous fungi in the middle of otherwise well-preserved and managed silages.

Many of the secondary metabolites produced by known contaminants of silage have not been analysed for in silage. It is thus possible that there are so far undetected metabolites playing a role in intoxications with silage. The list of possible contaminants is very long and silage is an extremely difficult matrix since it is full of organic acids, sugars, chlorophyll and numerous other small molecules, of which many cannot

be easily removed by, eg, reversed phase solid phase extraction. Very few methods in silage have been published so there is a need for high specificity methods like LC-MS/MS with at least two transitions or daughter ion scans.

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ORIGINAL MANUSCRIPT (II)

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Analysis of Moniliformin in Maize Plants Using Hydrophilic Interaction Chromatography

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A novel HPLC method was developed for detection of the *Fusarium* mycotoxin, moniliformin in whole maize plants. The method is based on hydrophilic interaction chromatography (HILIC) on a ZIC zwitterion column combined with diode array detection and negative electrospray mass spectrometry (ESI^--MS). Samples were extracted using acetonitrile–water (85:15), and the extracts were cleaned up on strong anion exchange columns. By this procedure we obtained a recovery rate of 57–74% moniliformin with a limit of detection at 48 ng/g and a limit of quantification at 96 ng/g using UV detection at 229 nm, which is comparable to current methods used. Limit of detection and quantification using ESI^--MS detection was 1 and 12 ng/g, respectively. Screening of maize samples infected with the moniliformin producing fungi *F. avenaceum, F. tricinctum*, or *F. subglutinans* detected moniliformin levels of 1–12 ng/g in 15 of 28 samples using ESI^--MS detection. To our knowledge this is the first example of HILIC separation in mycotoxin analysis.

KEYWORDS: Moniliformin; *Fusarium*; hydrophilic interaction chromatography; zwitterionic detection; HPLC; maize

INTRODUCTION

Moniliformin (**Figure 1**) is a frequently occurring mycotoxin in cereals and maize world wide (*1*), and produced by several *Fusarium* species including *F. avenaceum*, *F. proliferatum*, *F. subglutinans, F. tricinctum* and *F. verticillioides* (*2, 3*). Moniliformin has a weak cytotoxicity (*4*), but it has an acute toxicity comparable to that of other *Fusarium* derived mycotoxins, such as the type A trichothecenes diacetoxyscirpenol and T-2 toxin (*5*). Currently, the level of moniliformin is not regulated in food or feed in the EC, USA or any other country.

The mode of action of monilifomin has been linked to inhibition of enzyme systems and glucogenesis (*6*). Moniliformin has been suggested to be associated with Keshan disease, a human myocardial impairment occurring in areas of China with large consumption of moniliformin contaminated maize (*7, 8*), although others failed to link it to the disease (*9*). The symptoms of Keshan disease are similar to those of animals suffering from moniliformin contamination (*10*). Scandinavian studies have shown that grains from Finland and Norway contain up to 0.81 mg/kg and 0.95 mg/kg moniliformin (*11, 12*), respectively. Worldwide analysis of maize and grain samples have detected moniliformin levels of 2 mg/kg in Austrian cereal grains (*13*) and 3.2 mg/kg in Gambian and South African maize and maize products (*1*). During the last four years there have been numerous cases of ill thrift and health problems especially in dairy cows in Denmark, which has been claimed to be caused by mycotoxins in the maize silage used as feed. The problem has been associated with both *Penicillium* metabolites (*14, 15*) and/or *Fusarium* metabolites.

Surveys have shown that *F. avenaceum* is one of the most frequently observed species in Scandinavian cereal grains (*12, 16*). We therefore hypothesized that moniliformin could be formed in high levels in maize plants.

Determination of moniliformin is very different from other mycotoxins since it is a small, highly polar, acidic molecule with pK_a value of 0.5 (*17–19*). Subsequently, it is thus not well retained on reversed phase chromatography (RP), which is the most powerful HPLC separation mode as it gives very sharp peaks and is compatible with atmospheric pressure ionization mass spectrometric (MS) techniques such as electrospray ionisation (ESI). Positively charged ion-pairing reagent can be added to increase retention (*12, 20–22*), also for MS detection (*11, 16*). However it is our experience that such ion-pair reagent will impair positive ionization on the instrument for many weeks.

For some time we have used hydrophilic interaction chromatography (HILIC) for highly polar substances such as sugars and small acids since it can be interfaced with atmospheric pressure ionization-MS. However HILIC methods seem not to have been used for mycotoxins, even though many mycotoxins like nivalenol, patulin, moniliformin, 3-nitropropionic acid, and terrestric acid are quite difficult to separate by RP chromatography. The only HILIC-like method is that published for cyclopiazonic acid that resembles a mixed ion-exchange–ion-pair chromatographic method as the retention increased with increasing buffer concentration (*23*). HILIC has previously been used for detection of other highly polar compounds such as

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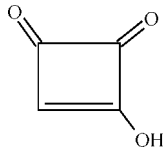

Figure 1. Structure of moniliformin.

carbohydrates, glycopeptides, nucleic acids, and shellfish toxins (*24, 25*).

Because we were unable to find any studies on the presence of moniliformin in whole maize plant parts, it was decided to develop a method for moniliformin for this. As a result of this we present this HPLC-HILIC-UV method with the possibility to combine with negative ESI-MS for the quantification of moniliformin in maize plants. The method uses a zwitterion stationary phase (ZIC-HILIC) column for analytical separation, and this is to our knowledge this is the first report of a HILIC method for a mycotoxin.

MATERIALS AND METHODS

Chemicals. All chemicals and standards were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were gradient grade and other chemicals analytical grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA).

Moniliformin was purchased as the sodium salt and a 73 μg/mL stock solution of moniliformin in acetonitrile (MeCN)–water (85:15) was made and stored at −20 °C. Purity was confirmed by HPLC-UV-MS using ESI^- and ESI^+ (*26*).

Identification of Viable Fusaria in the Maize Plant Parts. Chopped maize samples, 28 in all, were collected at harvest from different farms across Denmark. Four or five pieces of maize (5–10 mm × 10–30 mm) were placed on 10 Czapek-Dox iprodion dichloran agar (CzID) (*27*) plates per sample. The agar plates were incubated for 7 days at 25 °C below alternating black light and cool white light tubes (12 h on / 12 h off). The emerged *Fusarium* colonies were visually grouped. Colonies representing the different groups were isolated on potato dextrose agar (PDA) with 7 days incubation at 25 °C in darkness. For morphological identification the isolates were grown on PDA, yeast extract sucrose agar (YES) and Spezieller nährstoffarmer agar (SNA) with filter paper. Preparation of PDA, YES, and SNA has been described elsewhere (*28*). The isolated *Fusarium* cultures were identified morphologically (*28*).

Chemical Analyses. *Sample Preparation and Extraction.* Chopped maize plant pieces (5–10 mm × 10–30 mm) from a *Fusarium*-free sample also shown to be moniliformin-free by HPLC-MS were spiked in three replicas with 50 μL of pure solvent, or 0.96; 1.92; 5.72; or 11.5 μg moniliformin/mL to obtain spiked moniliformin levels of 0, 48, 96, 288, and 576 ng/g for HPLC-UV determination. The experiments were performed on three different days to establish day to day variation.

For HPLC-MS analysis spiked samples were prepared in the same way in levels of 3, 6, 12, and 24, 48, and 96 ng/g. Spiked maize samples were incubated at room temperature 30 min prior to extraction, allowing moniliformin to enter plant material. Spiked maize pieces, 4 g, were placed in four 5 mL cryo tubes together with 3 mL MeCN-water (85:15) and approximately 10 3 mm stainless steel balls. The maize pieces were then homogenized using a mini-bead beater (Biospec Products Inc.; Bartlesville, OK) for 1 min. The four 1 g subsamples were pooled in a 50 mL tube together with an additional 28 mL of MeCN-water (85:15). The mashed maize samples were extracted on a rotary shaker at 120 rpm for 30 min and filtered though Whatman no. 1 filters (Brentford, UK). Extracts, 20 mL equivalent to 2 g maize sample, were evaporated to dryness under a stream of nitrogen and dissolved in 2 mL methanol.

Moniliformin Detection of Naturally Contaminated Maize Samples. Chopped pieces of maize from the 28 samples used in the *Fusarium* analysis were analyzed for moniliformin contamination. Ten g maize sample were ground and extracted with 160 mL MeCN–water (85:15). A subsample, 32 mL (equivalent to 2 g maize sample), of the filtered extract was evaporated to dryness and dissolved in 2 mL methanol.

Solid Phase Extraction Clean Up. The SPE clean up was performed with Strata SAX strong anion exchange (500 mg) columns (Phenomenex, Torrance, CA), which were placed in a vacuum manifold. The clean up procedure was adapted from Filek and Lindner (*13*) with minor adjustments, as we excluded a water washing step due to loss of moniliformin from the column. The columns were sequentially activated with 2 mL methanol, 2 mL water, and 2 mL 0.1 M HCl. The dissolved maize extracts were then added to the SAX columns and allowed to percolate through by gravity. The cartridge was sequentially washed with 2 mL methanol–water (50:50) and 2 mL 0.1 M HCl. Moniliformin was eluted with 2 mL 1.0 M HCl, which was evaporated under a stream of nitrogen. The dried moniliformin fraction was dissolved in 100 μL MeCN–water (85:15) and transferred to a HPLC vial.

Several other SPE cartridges were also tested including Oasis MAX (strong anion-RP phase, Waters Milford, MA), but it was not possible to elute moniliformin from it using HCl, NaH_2PO_4 or NH_4OH. Moniliformin could, however, be eluted with ion-pair modifiers including tetrabutyl ammonium hydroxide and tetra methyl ammonium hydroxide. Because these ion-pair modifiers are not volatile we abandoned the use of Oasis MAX columns in our SPE clean up. We also tested Strata NH_2 (Phenomenex) columns from which we were able to elute moniliformin with HCl, NaH_2PO_4 or ammonium hydroxide. Moniliformin did not show stable recoveries from this column and was therefore abandoned. Strata-X-AW (weak anion exchanger-RP phase) columns were also tested but did not give cleaner extracts than the SAX columns.

HPLC-UV. The HPLC analyses were performed on an Agilent (Torrance, CA) 1100 HPLC system controlled by Chemstation v 1.01 B. The system was equipped with a diode array detector (DAD) containing a 6-mm flow-cell collecting approximately two UV/vis spectra per second from 190 to 900 nm with a bandwidth of 4 nm. Moniliformin was detected and quantified at 229 ± 2 nm and confirmed by the full UV spectrum, after background subtraction. Samples of 3 μL were injected.

Separation was made on a 150 × 4.6 mm i.d., 3.5 μm ZIC HILIC (SeQuant, Umeå, Sweden) column using flow of 0.5 mL/min and a linear gradient system of MeCN-water system starting at 5% water increasing this to 15% water in 15 min, then increasing flow to 1 mL/min and the water to 50% in 1 min keeping this for 4 min, then reverting to 5% water in 1 min and keeping this for 2 min and then decreasing the flow to 0.5 mL/min. The water was buffered with 100 mM ammonium formate (pH 6.4).

HPLC-MS Confirmation. For confirmation of positive samples as well as to increase sensitivity, selected samples were also analysed by HPLC coupled to high resolution mass spectrometry. This was done on a Micromass HPLCT orthogonal time of flight mass spectrometer (Micromass Manchester, UK) equipped with a Z-spray ESI source and a LockSpray probe (*29*). The HPLC system was the same as described above. Two gradient systems were used: same as for HPLC-UV; and a faster gradient system, starting with 12% 100 mM formic acid increasing this to 30% in 6 min and then increased to 50% in 1 min, maintaining this for 3 min, and then reverting to the start conditions in 5 min. Samples were analyzed in negative electrospray mode as described previously (*26*) except that the desolvation flow was 650 L/hr (nitrogen 99.9%) and that only one scan function was used with a potential difference of 17 V between the cones (skimmers). The $[M–H]^-$ ion at *m/z* 96.9926 was used for detection using an interval of m/z 96.9726–96.9946 (mass −0.02 amu to +0.002 amu, due to dead time correction of the MCP detector) (*29*).

Statistical Assessment. The relative standard deviations of spiked and pure samples quantified with HPLC-UV and HPLC-MS were determined using a linear calibration form.

RESULTS AND DISCUSSION

SPE. SAX columns have previously been applied successfully in SPE clean up procedures from maize and cereal grains (*1, 13, 21*). With this column we were able to elute moniliformin with HCl

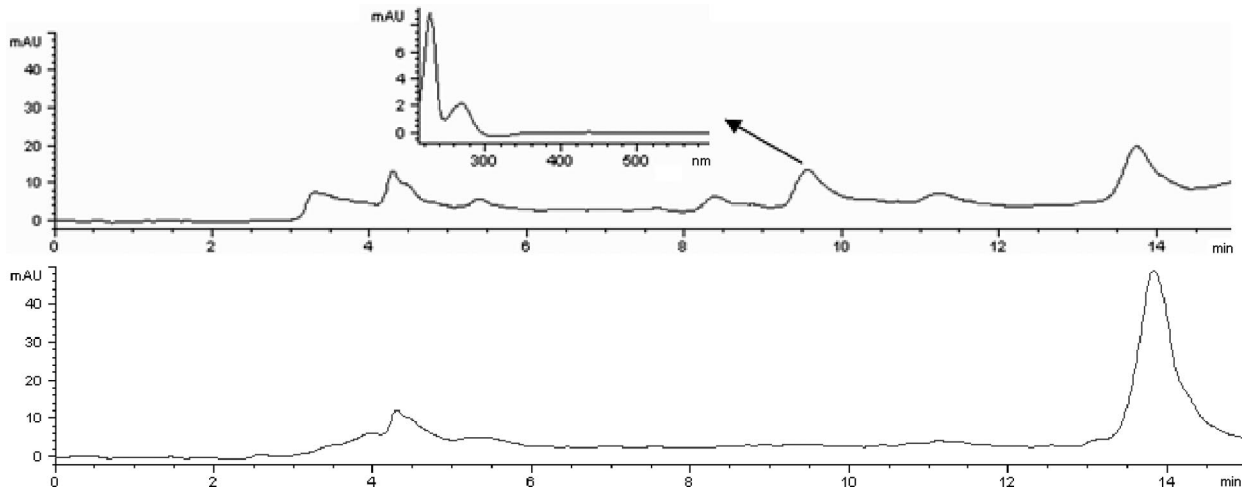

Figure 2. Chromatograms of maize sample spike with 288 ng/g moniliformin (top) and moniliformin free natural sample (bottom). Confirmation spectrum of moniliformin in the spiked sample is inserted.

and NaH_2PO_4 but not with NH_4OH. Because phosphate is not volatile we chose to use HCl as our elution reagent in our final protocol.

The present SPE clean up protocol was based on that of Filek and Lindner (*13*) who reported a consistent recovery of moniliformin in cereal grain samples of 70%. Likewise, from the maize samples spiked to contain moniliformin levels of 48, 96, 288, and 576 ng/g, moniliformin was recovered in a concentration independent manner ranging from 57 to 74% in our experiments indicating that the clean up procedure can be applied to the more complex maize matrix. No differences were observed in recovery rate between the three days, indicating a stable recovery.

HPLC-UV. The detection limit by HPLC-UV was approximately 48 ng/g ($s/n > 5$ at 229 nm, and full UV spectrum confirmation possible after background subtraction) and the quantification limit 96 ng/g (standard deviation from all three rounds of triplicate validation <20% at this level). This is comparable to currently used HPLC-UV methods, in which the quantification limit ranged from 20 to 120 ng/g (*12, 20, 30*).

When using a linear calibration R^2 was determined from the 0.994, 0.991, and 0.965 in spiked extracts from the three validation rounds (four levels in triplicate), 0.999 on the pure standards. For complete separation of moniliformin from UV interfering maize compound, a long run time of 15 min was used in HPLC-UV analysis. With this method moniliformin had a consistent retention time of 9.6–9.7 min in an area with little inference from maize compounds (**Figure 2**).

HPLC-MS. With MS detection we could detect moniliformin below 1 ng/g from spiked maize samples; however, limit of quantification was 12 ng/g (<20% standard deviation on lowest point). Moniliformin could be recovered from the samples spiked with 12–96 ng/g (four levels in triplicate) in a linear manner and a R^2 of 0.933. With the fast HILIC gradient program the retention time was 5 min with a runtime to 12 min. This could not be reduced as it was necessary to elute with 50% 100 mM formic acid to elute stronger retained compounds than moniliformin to avoid build up of contaminants on the column.

Since no qualifier ion was observed using in-source fragmentation, the mass accuracy was vital for specificity, which was demonstrated in two extracts where a peak partly coeluting at 9.55 min at *m/z* 96.56 was detected (moniliformin $[M\text{-}H]^-$ 96.9926 amu) when using a wide ion range; however, when using a narrow ion trace of *m/z* −0.02 to +0.002 this was not observed.

HILIC is not as sensitive as RP when using UV detection due to the broader peaks. However, when using HILIC-MS, the higher concentration of organic solvent at the point of elution gives a significantly better spray and thus compensates for the wider peaks compared to HPLC-RP-MS. This effect will probably vary between instruments. On our Micromass Z-spray source (Mark II) the same column in a 2 mm i.d. format tested at flows of 0.1–0.2 mL/min yielded far poorer sensitivity than the 4.6 mm i.d. column with a flow of 0.5 mL/min. Moniliformin showed very strong ionization, actually so strong that the TIC trace was lowered during peak elution (**Figure 3E**) in the high concentration samples indicating that it may not be very susceptible to ion-suppression. This was further supported by the same recovery rate obtained by ESI-MS and UV detection. However, the ESI tip became quite dirty during the run and had to be cleaned every 2 days, mainly due to the high flow rate of solvent into the source.

A 100 × 2 mm i.d., 3 μm polyhydroxyethyl aspartamide column was also tested for HILIC-MS, and from pure standards it did give better detection limits due to sharper peaks and lower buffer concentration needed for elution of moniliformin. However when analyzing real samples numerous impurities coeluted with moniliformin and obscured its detection even at the highest calibration levels.

Naturally Contaminated Samples. One or more *Fusarium* species could be isolated from all of the 28 examined maize samples. The predominant species were *F. avenaceum* and *F. graminearum*, which both were present in 13 samples (**Table 1**). *F. culmorum* and *F. equiseti* were also frequently isolated, occurring in 12 and 10 samples, respectively. Two isolates of *F. tricinctum* and three isolates of *F. subglutinans,* which are known moniliformin producers, were also identified.

On one hand, moniliformin could not be detected in any of the samples by HPLC-UV indicating that the contamination levels were below 48 ng/g. The samples were therefore analyzed by HPLC-MS and moniliformin was successfully detected in 15 out 28 samples; however, they were all below level of quantification. Moniliformin was detected in 11 of the 16 samples which contained *F. avenaceum, F. subglutinans*, and/or *F. tricinctum*, whereas it was only detected in 4 of the 12 samples without a potential moniliformin producing species. This indicates that the moniliformin producing fungi are unevenly distributed in the maize plant as the mycological examination of the randomly selected chopped maize pieces did not detect potential moniliformin

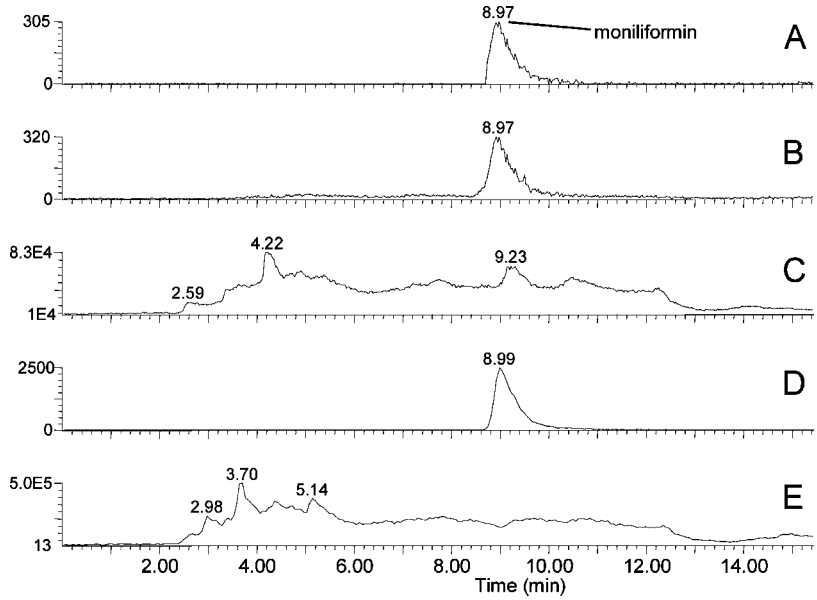

Figure 3. HPLC-ESI$^-$ chromatograms of anion exchanged maize plant extract: **A**, naturally contaminated with moniliformin (approx 10 ng/g) m/z 96.9726–96.9946 ([M-H]$^-$, −0.02 to +0.002 amu); **B**, the same but ± 0.5 amu; and **C**, same but TIC trace. **D**, spiked sample (96 ng/g) of [M-H]$^-$ (−0.02 to +0.002 amu); and **E** TIC of the same. Gradient system was the same as used for HPLC-UV.

Table 1. Presence of Fusarium Species and Moniliformin in 28 Maize Samples

F. avenaceum	*F. crookwellense*	*F. culmorum*	*F. equiseti*	*F. flocciferum*	*F. graminearum*	*F. sambucinum*	*F. subglutinans*	*F. sporotrichioides*	*F. tricinctum*	moniliformin (ng/g)
X										<12
X			X		X					<12
X		X								<12
X		X			X					<12
X		X				X				<12
X										nd
		X	X				X			nd
X		X								<12
X					X					nd
X				X	X					<12
					X					nd
X			X		X		X			<12
			X		X					nd
		X	X					X		nd
	X									<12
X		X	X							<12
					X			X		nd
X								X	X	nd
					X					<12
	X				X			X	X	<12
	X	X	X							nd
X		X								nd
								X		nd
		X			X			X		<12
		X	X							nd
			X		X					<12
		X	X							nd
	X						X			<12

producers. On the other hand, in most cases moniliformin-producing species were detected in moniliformin containing samples.

The low and presumable nontoxic levels quantities of moniliformin observed in the examined maize samples are due to the examination of maize pieces derived from the entire plant, whereas the high moniliformin levels in maize reported from other studies are mainly based on maize kernels. We examined moniliformin in samples derived from whole maize plants because maize in Denmark is primarily used as silage. Contamination studies of moniliformin solely in maize kernel is therefore not relevant for Danish grown maize.

This is to our knowledge the first report of HIILC based detection of a mycotoxin. Using this method we were able to for the first time to quantify moniliformin in samples derived from entire maize plants. With this method we are able to screen samples for moniliformin contamination above 48 ng/g using HPLC-UV detection or use HPLC-MS to detect moniliformin down to below 1 ng/g.

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Development of a LC-MS/MS Method for the Analysis of Enniatins and Beauvericin in Whole Fresh and Ensiled Maize

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A LC-MS/MS method for the detection of beauvericin and the four enniatins A, A1, B, and B1 in maize and maize silage was developed. The method uses direct injection of maize extracts without any tedious and laborious cleanup procedures. The limit of quantification was determined at 13 ng g^{-1} for beauvericin and at 17, 34, 24, and 26 ng g^{-1} for enniatins A, A1, B, and B1, respectively. The method was used in surveys of the compounds in fresh maize samples collected at harvest in 2005 and 2006. All samples had the same distribution of the enniatins: B > B1 > A1 > A. Enniatin B was present in 90% of the samples in 2005 and in 100% in 2006 at levels up to 489 and 2598 ng g^{-1}, respectively. Beauvericin contamination was more frequently detected in 2006 than in 2005 (89 and 10%, respectively) and in higher amounts (988 and 71 ng g^{-1}, respectively). The occurrence of beauvericin and the four enniatins was examined in 3-month-old maize silage stacks from 20 different farms. As observed in fresh maize, enniatin B was the most abundant compound in ensiled maize and was found from 19 stacks at levels up to 218 ng g^{-1}. The stability of enniatin B in maize silage was assessed by analyzing samples from 10 of the silage stacks taken after 3, 7, and 11 months of ensiling. Enniatin B could be detected at all locations after 11 months and appeared to be stable during ensiling.

KEYWORDS: Beauvericin; enniatins; *Fusarium*; maize; maize silage; LC-MS/MS

INTRODUCTION

Fusarium infections in maize are a global problem. This is also true in Denmark, where maize has become an important part of the diet of dairy cows. Besides the direct yield-reducing effects, *Fusarium* can also produce mycotoxins that are of great concern to Danish farmers. One of the most commonly occurring *Fusarium* species in maize and cereals in Scandinavia is *F. avenaceum* (*1*−*3*). This species is a known producer of enniatins A, A1, B, B1, B2, and B3 (*4*) and the structurally related beauvericin (*5*). Beauvericin and enniatins A, A1, B, and B1 are, however, receiving most of the attention as food contaminants. Enniatins and beauvericin are cyclic hexadepsipeptides produced by several species of *Fusarium* besides *F. avenaceum* (*5*−*10*). Several of these species are also present in maize (*2*) and may contribute to beauvericin and enniatin contamination. Enniatins and beauvericin consist of three *d*-2-hydroxycarboxylic acid and -*N*-methylamino acid residues linked alternately. Beauvericin and enniatins A, A1, B, and B1 differ in the substituents on the three L-*N*-methylamino acid residues (**Figure 1**). Enniatin A contains three *sec*-butyl substituents, whereas enniatin A1 contains two *sec*-butyl and one *iso*-propyl. Enniatin B contains three *iso*-propyl substituents, whereas enniatin B1 contains two *iso*-propyl groups and one *sec*-butyl. Beauvericin contains three aromatic phenylmethyl substituents.

Enniatins and beauvericin are cytotoxic (*4*) and toxic to insects (*11*), bacteria (*12*), and other fungi (*13*). The toxic effect has been linked to several mechanisms. The apolar nature of enniatins and beauvericin enables incorporation into cellular membranes in which they create cation selective channels (*12*) and thereby disturb the intracellular ionic homeostasis (*14, 15*). Enniatins and beauvericin have inhibitory effects on acyl-CoA: cholesterol acyltransferase (ACAT) (*16*) and on Pdr5p, a multidrug efflux pump in *Saccharomyces cerevisiae* (*17*). Enniatins and beauvericin are able to accumulate in poultry tissues, although only very small amounts have been detected (*18*).

Several methods for the detection of beauvericin and enniatins have been developed. Previously, HPLC with UV detection has been used to detect beauvericin and enniatins in naturally contaminated maize and cereal kernels (*19*−*21*). UV detection of beauvericin and enniatins is only possible at low wavelengths

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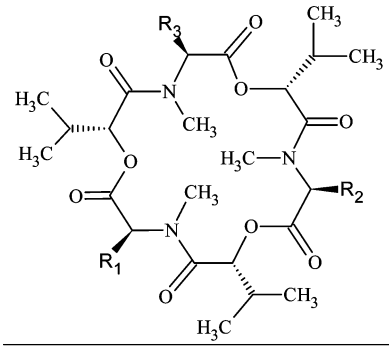

Enniatin A	R_1=R_2=R_3= -$CH(CH_3)CH_2CH_3$
Enniatin A_1	R_1=R_2= -$CH(CH_3)CH_2CH_3$, R_3= -$CH(CH_3)_2$
Enniatin B	R_1=R_2=R_3= -$CH(CH_3)_2$
Enniatin B_1	R_1=R_2= -$CH(CH_3)_2$, R_3= -$CH(CH_3)CH_2CH_3$
Beauvericin	R_1=R_2=R_3= -$CH_2C_6H_5$

Figure 1. Chemical structures of enniatins and beauvericin.

(192−209 nm), which makes this detection method vulnerable to interference from coeluting compounds. UV detection can therefore be applied only to relatively simple matrices such as grains and kernels, but is not applicable to complex matrices such as maize plants.

More recently, LC-MS/MS methods for the detection of beauvericin and enniatins in maize kernels and cereal grains have been developed (*22*−*25*). With the numerous heteroatoms in beauvericin and enniatins, they ionize very well in positive electrospray, making this an obvious choice for LC-MS/MS detection. Given that Danish maize is frequently infected by beauvericin- and enniatin-producing *Fusarium* species, we wanted to develop a method for the detection of these compounds in fresh and ensiled maize. Because nearly all Danish maize is used as cattle feed, the antibiotic properties of beauvericin and enniatins may impair the rumen microflora (*26*), which may lead to ill-thrift in the herds. We report the development of a fast method for the detection of beauvericin and enniatins A, A1, B, and B1 in fresh and ensiled maize without using solid-phase extraction cleanup procedures.

MATERIALS AND METHODS

Chemicals. Acetonitrile (MeCN) was of gradient grade and purchased from Sigma-Aldrich (St. Louis, MO). Ammonium formate (99.995+%) used in the mobile phase of the HPLC system was purchased from Sigma-Aldrich. Water was purified from a Milli-Q system (Millipore, Bedford, MA). Beauvericin was purchased from Sigma-Aldrich, whereas a mixture of enniatins A, A1, B, and B1 was a gift from Dr. Rainer Zocher, Technical University of Berlin. Two standard solutions were made in MeCN, one containing 100 μg/mL beauvericin and another containing 400 μg/mL of the four enniatins in total. On the basis of HPLC-UV quantification at 200 nm the distribution of the enniatins was as followd: A1, 34%; A, 17%; B, 24%; and B1, 26%.

Sample Preparation. Finely chopped maize pieces, 30 g (5−10 mm × 10−30 mm), from a *Fusarium*-free sample, thus assumed not to contain enniatins and beauvericin, were processed in a kitchen blender and extracted for 1.5 h with 480 mL of MeCN/H_2O (84:16) on a rotary shaker. The extract was filtered through a Whatman 1 filter (Brentford, U.K.). Extracts, equivalent to 0.25 g of maize, were spiked in triplicate with 100 μL of a 0, 0.25, 0.5, 1, 2, 4, 8, or 16 μg/mL enniatin mixture to obtain total levels of 0, 100, 200, 400, 800, 1600, 3200, or 6400 ng g^{-1}. The distribution of the different enniatins is shown in **Table 1**. The samples were also spiked with 100 μL of 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0, or 2.0 μg of beauvericin mL^{-1} to obtain levels of 0, 13, 25, 50, 100, 200, 400, and 800 ng g^{-1} (**Table 1**). One milliliter of the spiked extract was transferred directly to a HPLC vial and analyzed.

Table 1. Spike Levels of Enniatins and Beauvericin (ng g^{-1})[a]

enniatin A	0	17	34	67	134	268	537	1073
enniatin A1	0	34	67	134	269	537	1075	2149
enniatin B	0	24	48	95	190	380	760	1520
enniatin B1	0	26	52	104	207	414	829	1658
beauvericin	0	13	25	50	100	200	400	800

[a] Samples were spiked in triplicate on three different occasions.

Table 2. MS Method Including Scan Events, Retention Time (RT), Transition Ions, and the Cone and Collision Energies (CE) Used

compound	scan event	RT (min)	ion type	transition (m/z)[a]	ratio[b]	cone (V)	CE (V)
enniatin B	1	3.6	quantifier	640 → 196	5.7	100	27
			qualifier	640 → 527		100	20
enniatin B1	2	4.1	quantifier	654 → 196	2.8	100	25
			qualifier	654 → 228		100	25
enniatin A1	3	4.7	quantifier	668 → 210	5.5	100	25
			qualifier	668 → 541		100	20
enniatin A	4	5.2	quantifier	682 → 210	4.8	100	25
			qualifier	682 → 555		100	20
beauvericin	4	5.4	quantifier	784 → 244	22.6	100	25
			qualifier	784 → 362		100	25

[a] All transitions were made from $[M + H]^+$. [b] Average ratio of quantifier and qualifier ions in spiked samples.

Samples Collected at Harvest. Thirty samples, each weighting approximately 1 kg, of chopped maize were collected directly at harvest in late autumn of 2005 and 43 samples at harvest in 2006. Seven maize kernel samples were also collected at harvest in 2006. The samples were stored at −20 °C before extraction. Ten grams of each sample was ground and extracted with 160 mL of MeCN/water (84:16). The extracts were filtered before 1 mL of each sample was transferred to HPLC vials.

Samples Collected during Ensiling. Samples of approximately 1 kg were collected from 20 different silage stacks after 3 months of ensiling. The silage stacks were made from whole maize harvested in 2006 at different farms. The samples were taken 20 cm behind the cut surface of the horizontally placed silage stacks with a vertical drill. Samples from 10 of these stacks were also taken after 7 and 11 months of ensiling. The silage samples were treated in the same way as the samples collected at harvest.

HPLC-MS/MS. Liquid chromatography was performed on an Agilent (Torrance, CA) 1100 HPLC system controlled by MassLynx V4.1. Extracts of 1 μL were injected and separated on a Gemini C_6-Phenyl 3 μm 2 mm i.d. × 50 mm column (Phenomenex) using a constant flow of a 0.3 mL/min MeCN/water gradient starting at 55% MeCN, which was increased linearly to 100% in 7 min. The column was washed with 100% MeCN for 2 min at 0.5 mL/min before reverting to the 55% MeCN in 1 min, maintaining this for 5 min. The water contained 20 mM ammonium formate. The LC was coupled to a triple-quadrupole mass spectrometer (Waters-Micromass, Manchester, U.K.) with Z-spray ESI operated in positive mode source using a flow of 700 L/h nitrogen at 350 °C; hexapole 1 was held at 50 V. The system was controlled from MassLynx 4.1 (Waters-Micromass). Nitrogen was also used as collision gas, and the MS operated in MRM mode (dwell time = 200 ms) with the parameters shown in **Table 2**.

Enniatins and beauvericin in all samples were quantified with QuanLynx (Waters-Micromass). The limit of quantifications (LOQ) for beauvericin and enniatins A, A1, B, and B1 were for practical reasons set as the minimum calibration points (**Table 1**), which were 13, 17, 34, 24, and 26 ng g^{-1}, respectively. The quantifier ions of beauvericin and enniatins A, A1, B, and B1 had average signal-to-noise (S/N) ratios

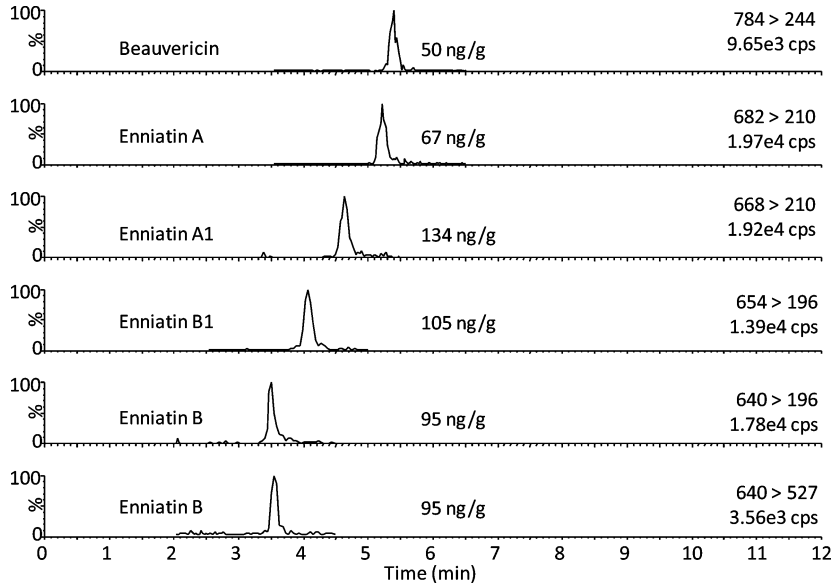

Figure 2. Extracted chromatograms of the quantifier ions from a fresh maize sample spiked with 50, 67, 134, 104, and 95 ng g^{-1} beauvericin and enniatins A, A1, B1, and B, respectively. The chromatogram of the qualifier ion of enniatin B is also shown. Peak heights of the ion transitions are given as counts per second (cps) and percent.

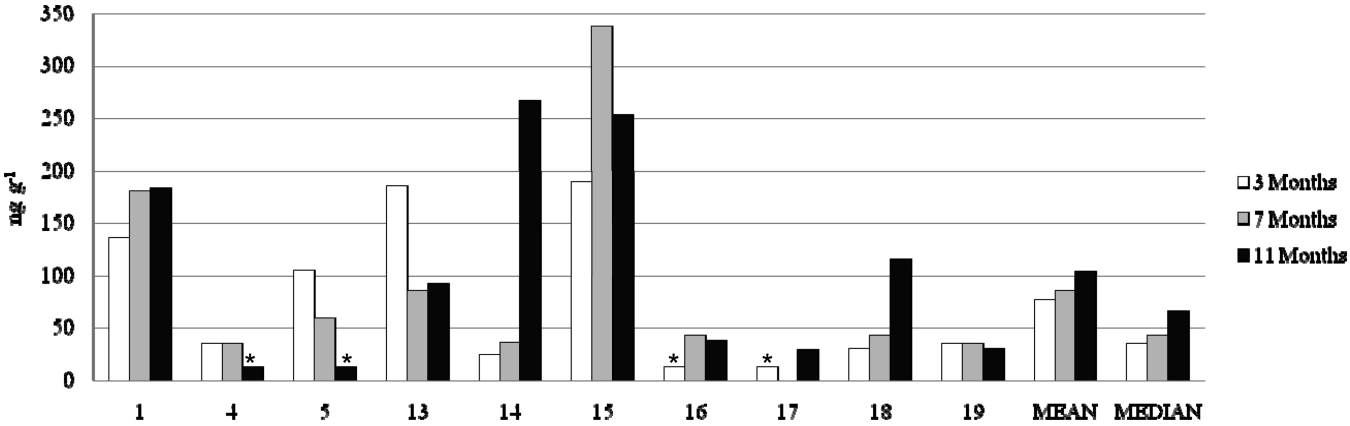

Figure 3. Content of enniatin B in 10 different maize silage stacks collected after, respectively 3, 7, and 11 months of ensiling. An asterisk indicates trace amounts below LOQ. Means are calculated by setting samples in which the compounds were not detected to 0 and trace values below LOQ to LOD.

of 19, 18, 32, 21, and 19, respectively. The limits of detection (LOD) for the compounds were calculated as points having S/N ratios of 10. The estimated LODs for beauvericin and enniatins A, A1, B, and B1 were 7, 9, 10, 12, and 13 ng g^{-1}, respectively. Concentrations of enniatins and beauvericin in natural samples were calculated on the basis of standard curves made from spiked samples. The average peak areas from the blank samples were subtracted from the peak area of the spiked samples before recoveries were calculated and standard curves plotted.

RESULTS AND DISCUSSION

Method Development. Ammonium formate was used in the water, which resulted almost exclusively in the formation of $[M + NH_4]^+$, which was fragmented between the cone (skimmer 2) and hexapole 1 to $[M + H]^+$. Collision energy was optimized for the two major daughter ions of each compound. The masses of the quantifier ions corresponded to [monomer with phenylmethyl residue + H − H_2O]$^+$ for beauvericin, [monomer with *sec*-butyl residue + H − H_2O]$^+$ for enniatins A and A1, and [monomer with *iso*-propyl + H − H_2O]$^+$ for enniatins B and B1 (**Table 2**). These fragments have been used as quantifier (*22*) or qualifier (*24*) in other tandem MS methods.

Liquid chromatography separation of enniatins and beauvericin was performed with a Gemini C_6-phenyl column with which we were able to develop a fast method with baseline separation of the four enniatins (**Figure 2**). The column has a good ability to retain aromatic compounds such as beauvericin, and with the column we were able to let beauvericin elute after the last enniatin, enniatin A. Beauvericin coelutes normally together with either enniatin B or B1 on C_{18} columns (*22*−*24*), but having beauvericin eluting after the enniatins may improve sensitivity to beauvericin as enniatins B and B1 usually occur in higher amounts than enniatin A in natural samples.

Validation. A beauvericin- and enniatin-free maize sample was spiked with seven levels of beauvericin and enniatins in triplicate on three different occasions. The spiked samples were analyzed on each occasion and recoveries calculated (**Table 3**).

The five compounds were recovered linearly on all three occasions with only little variation between the different experiments with R^2 values ranging from 0.994 to 0.999. No suppression matrix effects on the recovery effects were observed; in fact, the maize matrix seemed to enhance the signal of beauvericin and enniatins, resulting in recoveries above 100% in many of the samples.

Table 3. Recovery of Enniatins and Beauvericin from Spiked Maize Samples on Three Different Days

day	compound	n^a	spike level (ng g^{-1})	recovery (min−max)	SD[b]	R^2
1	enniatin A	21	17−1073	102 (76−116)	10	0.999
	enniatin A1	21	34−2149	97 (76−136)	12	0.998
	enniatin B	21	24−1520	107 (86−130)	13	0.997
	enniatin B1	21	26−1658	108 (88−137)	12	0.996
	beauvericin	21	13−800	104 (87−145)	14	0.998
2	enniatin A	21	17−1073	103 (91−123)	8	0.998
	enniatin A1	21	34−2149	103 (80−130)	11	0.999
	enniatin B	21	24−1520	109 (95−151)	14	0.999
	enniatin B1	21	26−1658	110 (86−173)	26	0.998
	beauvericin	21	13−800	103 (68−132)	13	0.999
3	enniatin A	21	17−1073	93 (77−127)	15	0.994
	enniatin A1	21	34−2149	91 (77−117)	8	0.995
	enniatin B	21	24−1520	96 (78−121)	13	0.997
	enniatin B1	21	26−1658	100 (80−134)	19	0.995
	beauvericin	21	13−800	96 (79−114)	11	0.997

[a] Number of samples. [b] Standard deviation.

Table 4. Occurrence and Content of Enniatins and Beauvericin in Whole Maize in 2005 and 2006 at Harvest[a]

	compound	n^b	positive (%)	mean[c] (ng g^{-1})	median (ng g^{-1})	range (ng g^{-1})
2005	enniatin A	30	3	0	nd[d]	nd−<17
	enniatin A1	30	10	1	nd	nd−<34
	enniatin B	30	90	124	75	nd−489
	enniatin B1	30	47	9	nd	nd−79
	beauvericin	30	10	4	nd	nd−73
2006	enniatin A	43	12	6	nd	nd−106
	enniatin A1	43	35	13	nd	nd−107
	enniatin B	43	100	366	204	<24−2598
	enniatin B1	43	84	81	44	nd−496
	beauvericin	43	98	116	32	nd−988
grains	enniatin A	7	0	0	nd	nd
	enniatin A1	7	29	3	nd	nd−<34
	enniatin B	7	86	577	539	nd−1627
	enniatin B1	7	86	89	79	nd−235
	beauvericin	7	71	94	23	nd−496

[a] Data for maize grain samples from the harvest 2006 are also shown. [b] Number of samples. [c] Means are calculated by setting samples in which the compounds were not detected to 0 and trace values below LOQ to LOD. [d] nd, not detected.

Fresh Maize. Beauvericin and enniatins in 30 maize samples collected at harvest in autumn 2005 and 43 in 2006 were analyzed (**Table 4**). The results from the analysis showed that the enniatins occurred in a ratio of B > B1 > A1 > A in both years. Beauvericin and all four enniatins were more frequently detected in 2006 samples than in 2005 samples and in higher amounts. Enniatin B was the most abundant in both years, occurring in 90% in 2005 and in 100% in 2006, ranging up to 489 and 2598 ng g^{-1}. The most notable difference between the two years was observed with beauvericin, which was rare in 2005 (10%, maximum = 73 ng g^{-1}), whereas it was a frequent contaminant in 2006 (84%, maximum = 988 ng g^{-1}). Seven samples from maize kernels collected at harvest in 2006 were also analyzed. The enniatins were distributed in the same patterns as in the maize samples, with enniatin B being the predominant compound.

The difference in beauvericin and enniatin contamination between the two years may be caused by climatic differences. The summer and autumn were warmer and wetter in 2006 than in 2005 (*27, 28*). The combination of warm and wet weather may be beneficial for infection of some species of *Fusarium*.

Table 5. Occurrence and Content of Enniatins and Beauvericin in 3-Month-Old Maize Silage Made from Whole Maize Harvested in 2006

compound	n^a	positive (%)	mean[b] (ng g^{-1})	median (ng g^{-1})	range (ng g^{-1})
enniatin A	20	0	0	nd	nd
enniatin A1	20	0	0	nd	nd
enniatin B	20	95	73	35	nd−218
enniatin B1	20	40	10	nd	nd−48
beauvericin	20	25	8	nd	nd−63

[a] Number of samples. [b] Means are calculated by setting samples in which the compounds were not detected to 0 and trace values below LOQ to LOD.

In a study of the *Fusarium* species in Danish maize we observed only *F. verticillioides* in samples from 2006 (data not shown). This species is normally found in areas with a warmer climate than in Denmark (*29*), but it apparently was able to infect Danish maize in 2006. *F. verticillioides* is an important producer of beauvericin, and higher incidence of beauvericin may be attributed to this species. The contamination levels of beauvericin and enniatins that we found were similar to levels found in cereal grains (oats, barley, and wheat) from other Scandinavian countries, although beauvericin in 2006 was slightly higher (*1, 3*).

Maize Silage. Beauvericin and the four enniatins were analyzed with the developed method in 20 samples from 3-month-old silage stacks. The silage stacks were made from whole maize harvested in autumn 2006. The samples contained less beauvericin and enniatins than the fresh maize samples, with enniatins A and A1 being absent (**Table 5**). As noted before, enniatin B was the predominant compound, occurring in 95 of the samples, ranging up to 218 ng g^{-1}. The beauvericin and enniatins in the ensiled maize samples are most likely produced by *Fusarium* while the plants were growing in fields, because we were not able to isolate any species of *Fusarium* from the silage samples.

Stability in Silage Stacks. The stability of enniatin B was examined in 10 silage stacks by analyzing samples collected in 3-, 7-, and 11-month-old silage stacks (**Figure 3**). Lactic acid bacteria, which are responsible for the ensiling process, have been shown to be able to bind or transform other *Fusarium* mycotoxins such as trichothecenes, zearalenone, and fumonisins (*30, 31*). For proper risk assessment it is important to examine how beauvericin and enniatins are conserved in the silage stacks. Enniatin B was chosen to represent the group of enniatins and beauvericin as it was the most abundant compound. The results show that enniatin B is very stable in the silage stacks and was present in all stacks after 11-month-old silage. The results did not show a consistent trend: three locations had the highest amounts after 3 months, three locations after 7 months, and four after 11 months. Samples from stacks 1 and 15 contained high amounts of enniatin B at all time points, whereas samples from stacks 4, 16, 17, and 19 contained low amounts of enniatin B. The average and median showed a small increase in enniatin B as the silage got older. These results suggest that the enniatins are not degraded in the period tested. When the variations between the time points in **Figure 3** are compared, it must be taken into account that the sampling procedure used in this study may have caused some of the observed differences in concentration levels due to the inhomogeneous maize silage matrix in which the enniatins are unevenly distributed.

There may be a degradation of enniatin formation in the first 3 months of ensiling because the enniatin contents in ensilaged maize were lower than in fresh maize. The most drastic changes occur within the first months of ensiling, with lactic acid bacteria transforming carbohydrates into lactic acid and thereby lowering

the pH. The microbes and environmental conditions will then stabilize after the first 3 months and remain at a consistent level for the remaining ensiling period. It is therefore possible that if enniatins are degraded or transformed by microbes such as lactic acid bacteria, this will occur during the first months of ensiling.

Conclusion. We have developed an easy method for fast simultaneous quantification of beauvericin and enniatins A, A1, B, and B1, which worked well in maize and maize silage. Enniatin B was the predominant enniatin in fresh maize and appeared to be stable during ensiling over the period examined.

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Potato carrot agar with manganese as an isolation medium for *Alternaria*, *Epicoccum* and *Phoma*

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ABSTRACT

A semi-selective medium for isolation of *Alternaria* spp., *Epicoccum* sp. and *Phoma* spp. from soil and plant samples was developed. The basal medium was a modified potato carrot agar (PCA), containing 10 g/L of potato and carrot. It is known that the target genera sporulate well on standard PCA when grown at 25 °C with an alternating light/dark cycle consisting of 8 h of cool-white daylight followed by 16 h darkness. Addition of 1.5% $MnCl_2$ 4 H_2O (w/v) inhibited most other fungi than *Alternaria*, *Epicoccum* and *Phoma* species when tested on pure cultures. The mycobiota of two soil samples and eight grain samples were examined using PCA-Mn and three commonly used isolation media, DRYES, DG18 and V8. On the three conventional media growth of several genera was observed with the predominant being *Aspergillus*, *Eurotium*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus*. Of these only *F. oxysporum* and *F. verticillioides* were able to grow on PCA-Mn. *Alternaria infectoria* and *Epicoccum nigrum* were present in three cereal grain samples, but emerged to a far lower degree on the three conventional media compared to PCA-Mn. Three black spored fungi, identified as *Phoma eupyrena*, *Paraconiothyrium minitan* and one unknown species, were isolated from the two soil samples when incubated on PCA-Mn but were absent on the three conventional media.

1. Introduction

The black mould genera *Alternaria*, *Epicoccum* and *Phoma* contain a wide range of pathogenic and saprobic species that occur worldwide in many different habitats. The anamorphic genus *Alternaria* comprises nearly 300 described taxa (Simmons, 2007), whereas the anamorphic genus *Phoma* includes more than 220 specific and intraspecific taxa (Boerema et al., 2004). The actual number of taxa may within the genus *Phoma* probably be much higher, as only a fraction of the thousands of species described in literature have been verified *in vitro* (Aveskamp et al., 2008). Currently there is only one species accepted of the genus *Epicoccum*, *E. nigrum* (Domsch et al., 2007) whose closest relatives are found within the *Phoma* genus (Arenal et al., 2000). The pathogenic species of the three genera can cause losses in agriculture by reducing crop yield in the field and through spoilage during storage. Small-spored *Alternaria* spp. are among the most predominant species found on cereals (Andersen et al., 1996; Kosiak et al., 2004), whereas one of the most important *Phoma* pathogens, *P. andigena* found on potato, is on the European Plant Protection Organisation (EPPO) list of quarantine organisms (EPPO/CABI, 1997). As saprotrophs, species of the three genera can be found on buildings and monuments (Gravesen et al., 1999; Gómez-Alarcón et al., 1995), wood (Kim et al., 2007, 2005), marine waters (Arvanitidou et al., 2000; Osterhage et al., 2000), cinematographic films (Abrusci et al., 2005) and several other habitats including *Alternaria* infections in human soft tissue (de Hoog and Horre, 2002).

The three genera *Alternaria*, *Epicoccum* and *Phoma* are rich sources of metabolites, which may be mycotoxins, allergens or host specific toxins (Shephard et al., 1991; Montemurro and Visconti, 1992; Domsch et al., 2007). Particularly metabolite production of *Alternaria* spp. has been extensively studied (Andersen et al., 2001, 2002) and more than 70 known metabolites with varying toxic and allergenic properties have been identified (Montemurro and Visconti, 1992).

Production of mycotoxins in food and animal feed is of concern to consumers and because each species has a specific profile of mycotoxins it is important to get an overview of fungal contamination for proper risk assessment. It is, however, problematic to isolate the fungi of interest due to competing fungi that may grow faster or produce antagonistic compounds. To circumvent this problem, semi- or fully selective media are desirable. A semi-selective medium for isolation of small-spored *Alternaria* containing dichloran, chloramphenicol, bacteriological peptone agar (DCPA) was developed by Andrews and Pitt (1986). The medium reduced the growth of *Aspergillus*, *Penicillium* and Zygomycetes, but these fungi would still emerge in samples where they dominated over *Alternaria* and thereby hamper numeration and isolation. An acidified weak potato-dextrose agar (AWPDA) with thiabendazole for isolation of small-spored *Alternaria* was developed by Hong and Pryor (2004). The medium inhibited growth of *Aspergillus*, *Fusarium* and *Penicillium* completely, while having only mild effects on *Alternaria*. The medium did, however, not have reducing effects on Zygomycetes,

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which, due to their fast growth, are among the most troublesome groups when isolating small black spored fungi, including *Alternaria*. Because these two media have some minor drawbacks, a need for a new isolation medium for isolation of small black spored fungi still exists. The purpose of this study therefore was to develop a simple medium for isolation of *Alternaria* spp., *E. nigrum* and *Phoma* spp. using potato carrot agar as base and manganese (II) chloride as the major selectivity factor.

2. Materials and methods

2.1. Fungal strains

The fungal strains used in this study are listed in Table 1. *Penicillium roqueforti*, *Pen. expansum*, *Aspergillus flavus*, *Asp. niger*, *Fusarium graminearum*, *F. verticillioides* and *Mucor* sp. were used to represent undesired genera. *Alternaria arborescens*, *A. infectoria*, *A. tenuissima*, *Epicoccum nigrum* and *Phoma pomorum* were used as representatives of target genera. Most of the strains originate from Danish maize, because PCA-Mn was developed as a medium for isolation of small black spored fungi from Danish maize samples.

2.2. Development of the isolation medium

The base of the isolation medium was a modified potato carrot agar (PCA) containing 10 g carrot and 10 g potato/L medium which is half strength of a standard PCA (Simmons, 1992). The basal medium was prepared with 20 g agar (SoBiGel, Bie & Berntsen, Roedovre, Denmark) and autoclaved in a 900 ml volume to which 100 ml autoclaved manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4\ H_2O$; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added after cooling to 55 °C and then poured immediately into sterile 90 mm Petri dishes. No antibiotics were added. In the early development stages three concentrations of $MnCl_2 \cdot 4\ H_2O$ (1, 2 and 3% (w/v)) was tested. The optimal concentration of $MnCl_2 \cdot 4\ H_2O$ was found to be 1.5% (w/v), which was used in the further experiments referred to as PCA-Mn.

Table 1
Fungal isolates used in the experiments

Analysis #	Species	Origin	Artificial soil[b]	Identification #[a]
1	*Aspergillus flavus*			IBT 13446
2	*Asp. niger*		1, 2	IBT 13460
3	*Mucor* sp.	Maize, DK	5, 6	IBT 41391
4	*Penicillium expansum*			IBT 6110
5	*Pen. roqueforti*		1, 2	IBT 16947
6	*Alternaria arborescens* sp-grp.	Maize, DK		IBT 41385
7	*A. arborescens* sp-grp.	Maize, DK		IBT 41386
8	*A. arborescens* sp-grp.	Maize, DK		IBT 41387
9	*A. infectoria* sp-grp.	Maize, DK		IBT 41388
10	*A. infectoria* sp-grp.	Maize, DK	1, 3, 5	IBT 41389
11	*A. infectoria* sp-grp.	Maize, DK		IBT 41390
12	*A. tenuissima* sp-grp.	Maize, DK	1, 3, 5	IBT 41382
13	*A. tenuissima* sp-grp.	Maize, DK		IBT 41383
14	*A. tenuissima* sp-grp.	Maize, DK		IBT 41384
15	*Epicoccum nigrum*	Maize, DK		IBT 41379
16	*E. nigrum*	Maize, DK		IBT 41380
17	*E. nigrum*	Maize, DK		IBT 41381
18	*Fusarium graminearum*	Maize, DK		IBT 41391
19	*F. graminearum*	Maize, DK		IBT 41392
20	*F. graminearum*	Maize, DK		IBT 41393
21	*F. verticillioides*	Maize, DK		IBT 41110
22	*F. verticillioides*		3, 4	IBT 9400
23	*F. verticillioides*			IBT 9492
24	*Phoma pomorum*	Maize, DK	2, 4, 6	IBT 41376
25	*P. pomorum*	Maize, DK		IBT 41377
26	*P. pomorum*	Maize, DK		IBT 41378

[a] Isolate number in the IBT collection, Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark.
[b] Selected strains were inoculated to sterile soil in six different combinations.

2.3. Selectivity test on pure cultures

Aspergillus spp. and *Penicillium* spp. were grown on Czapek yeast autolysate agar (CYA) (Samson et al., 2004), *Fusarium* spp. on potato dextrose agar (PDA) (Samson et al., 2004), *Mucor* sp. on V8 juice agar (V8) (Simmons, 1992) and *Alternaria* spp., *P. pomorum* and *E. nigrum* on PCA before the experiments. The fungi were then transferred as three point inoculation to PCA and PCA-Mn and incubated in two layers for 7 days at 25 °C with a an alternating light/dark cycle consisting of 8 h of cool-white daylight followed by 16 h darkness.

2.4. Artificial soil test

To test the isolation medium on complex samples with known fungal composition, six combinations of fungal spore suspensions were added to dried autoclaved soil (Table 1). *Asp. niger* and *Pen. roqueforti* were grown on CYA, *F. verticillioides* on Spezieller nährstoffarmer agar (SNA) (Nirenberg, 1976), *Mucor* sp., *A. infectoria*, *A. tenuissima* and *P. pomorum* on PCA before harvesting the spores. Spores were collected using sterile loop (QuadLoop, Miniplast; EIN-SHEMER, Post Menashe, Israel) and suspended in 3 ml sterile water. 200 µl of each spore suspension was added to 200 ml of the respective soils. Small portions of the soil samples were spread on five plates of five different media: czapek dox iprodione dichloran agar (CZID) (Abildgren et al., 1987), dichloran rose bengal yeast extract sucrose agar (DRYES) (Frisvad, 1983), dichloran 18% glycerol agar (DG18) (Hocking and Pitt, 1980), V8 and PCA-Mn. DRYES and DG18 plates were incubated for 7 days at 25 °C in the dark, whereas CZID and V8 plates were incubated under a 12-h dark/12-h light regime. PCA-Mn plates were incubated as described in the selectivity test of pure cultures.

2.5. Natural samples test

Ten different substrata were used to test the performance of the isolation medium in unknown and possibly complex natural matrixes. The chosen substrata were: camarque red rice (*Oryza sativa*, France), wild rice (*Zizania aquatica*, USA), a pepper mix [black, white and green pepper (*Piper nigrum*), pink pepper (*Schinus terebinthifolius*), Szechwan pepper (*Zanthoxylum piperitum*), allspice (*Pimenta dioica*) and coriander seeds (*Coriandrum sativum*)] (unknown origin), whole organic spelt grain (*Triticum spelta*, Hungary), whole wheat grain (*Triticum aestivum*, Denmark), whole barley grain (*Hordeum vulgare*, Denmark), soil (Uganda), soil (Denmark), organic red lentils (*Lens culinaris*, Turkey) and sunflower seeds (*Helianthus annuus*, unknown origin). Ten plates of the five different media used in the artificial soil test were used for each sample. The two soil samples were spread on the plates with a sterile loop. For the remaining samples, ten seeds were placed on each plate and allowed to settle on the agar plates over night before being moved to the incubation rooms. The samples were incubated as described before.

After one week of incubation, the emerged fungi were grouped based on colour, growth and appearance. The groups were then counted and one representative of each group was isolated. The fungi were isolated on the same media from which they were derived. Subsequently, the isolated cultures were grown on various media for species identification. *Eurotium* spp. were grown on Czapek agar with either 20% or 40% sucrose at 15 °C, 25 °C and 37 °C for 1 week in the dark. *Penicillium* spp. and *Aspergillus* spp. were grown on CYA, malt extract agar (MEA) (Samson et al., 2004), creatine sucrose agar (CREA) (Samson et al., 2004) and yeast extract sucrose agar (YES) (Samson et al., 2004) in dark for 1 week at 25 °C

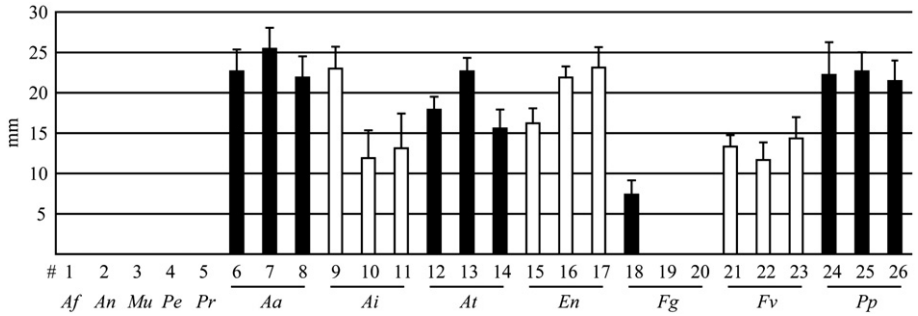

Fig. 1. Average growth of the isolates on PCA-Mn. Error bars indicates standard deviation. Af: *Asp. flavus*, An: *Asp. niger*, Mu: *Mucor* sp., Pe: *Pen. expansum*, Pr: *Pen. roqueforti*, Aa: *A. arborescens* sp-grp, Ai: *A. infectoria* sp-grp., At: *A. tenuissima* sp-grp., En: *E. nigrum*, Fg: *F. graminearum*, Fv: *F. verticillioides*, Pp: *P. pomorum*.

and on CYA at 37 °C for 1 week. *Fusarium* spp. were grown on PDA, YES and SNA with the various conditions described above. *Alternaria* spp. were grown on PCA and DRYES and *Phoma* and *E. nigrum* on MEA.

3. Results

3.1. Medium development

In the early development process three concentrations of $MnCl_2 \cdot 4\ H_2O$ (1, 2 and 3% (w/v)) were used in the potato carrot agar (PCA) and tested to find the optimal concentration. The preliminary results showed that most of the undesired species were unable to grow on 2% $MnCl_2 \cdot 4\ H_2O$, except for *Fusarium verticillioides*, which was also able to grow on 3%. All target isolates were able to grow on 3% $MnCl_2 \cdot 4\ H_2O$, except *Alternaria infectoria*, which also grew poorly on 2%. As a compromise a concentration of 1.5% (w/v) $MnCl_2 \cdot 4\ H_2O$ was chosen for further experiments in the medium referred to as PCA-Mn.

Pure cultures of target genera and of undesired genera were used to demonstrate the selectivity of PCA-Mn. Five strains, *Alternaria arborescens*, *A. infectoria*, *A. tenuissima*, *Epicoccum nigrum* and *Phoma pomorum*, were chosen as representatives of the three target genera, while *Aspergillus flavus*, *Asp. niger*, *Penicillium roqueforti*, *Pen. expansum*, *Mucor* sp., *Fusarium graminearum* and *F. verticillioides* were chosen to represent the undesired fungi that often hamper the isolation of *Alternaria*, *Epicoccum* and *Phoma*. The results of these experiments showed that all isolates of the target species grew well on PCA-Mn, whereas of the undesired species only one isolate of *F. graminearum* and all three *F. verticillioides* isolates were able to grow (Fig. 1). All species of all three target genera sporulated on PCA-Mn and their sporulation morphology were comparable to those on standard PCA.

3.2. Artificially inoculated soil samples

Six soil samples containing six combinations of target genera and undesired competing genera (Table 1) were made to demonstrate the usefulness of the medium in controlled complex samples. Soil samples 1 and 2 contained *Asp. niger*, *Pen. roqueforti* in combination with either *P. pomorum* or *A. infectoria* and *A. tenuissima*. The results showed that growth of *A. infectoria*, *A. tenuissima*, and *P. pomorum* were suppressed by *Asp. niger* and *Pen. roqueforti* on V8, DRYES and DG18, with *Asp. niger* dominating. On PCA-Mn, only growth of *A. infectoria*, *A. tenuissima*, *P. pomorum* were observed, whereas growth of *Asp. niger* and *Pen. roqueforti* were not observed. Soil samples 3 and 4 containing *Mucor* sp. in combination with either *P. pomorum* or *A. infectoria* and *A. tenuissima* were made to test how a fast growing Zygomycetes perform against the target genera. As observed before, growth of *A. infectoria*, *A. tenuissima* and *P. pomorum* were suppressed on V8, DRYES and DG18, but were the only species able to grow on PCA-Mn. Soil samples 5 and 6 contained *F. verticillioides* together with either *P. pomorum* or *A. infectoria* and *A. tenuissima*. *Fusarium verticillioides* dominated completely on V8, DRYES and DG18, but was completely absent on PCA-Mn in the soil sample with *P. pomorum*. In the soil sample with *F. verticillioides*, *A. infectoria* and *A. tenuissima* all three species were equally distributed on PCA-Mn.

3.3. Naturally infected samples

Ten samples of various ecology and geography were examined using the same medium as in the artificial soil tests. The aims of this experiment were to identify genera which can and which genera cannot grow on PCA-Mn. The usefulness of PCA-Mn as an isolation media for *Alternaria*, *Epicoccum* and *Phoma* in complex samples was also examined

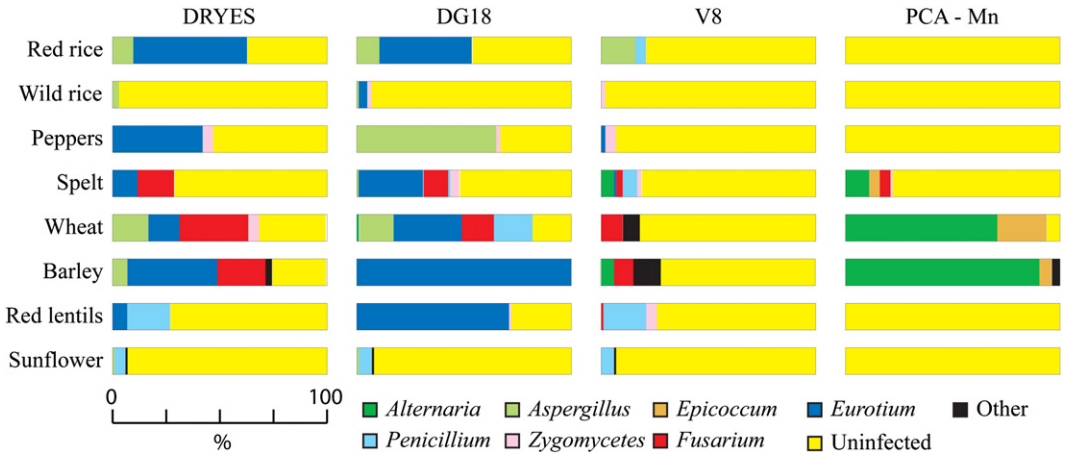

Fig. 2. Distribution of fungi isolated with DRYES, DG18, V8 or PCA-Mn from eight grain samples.

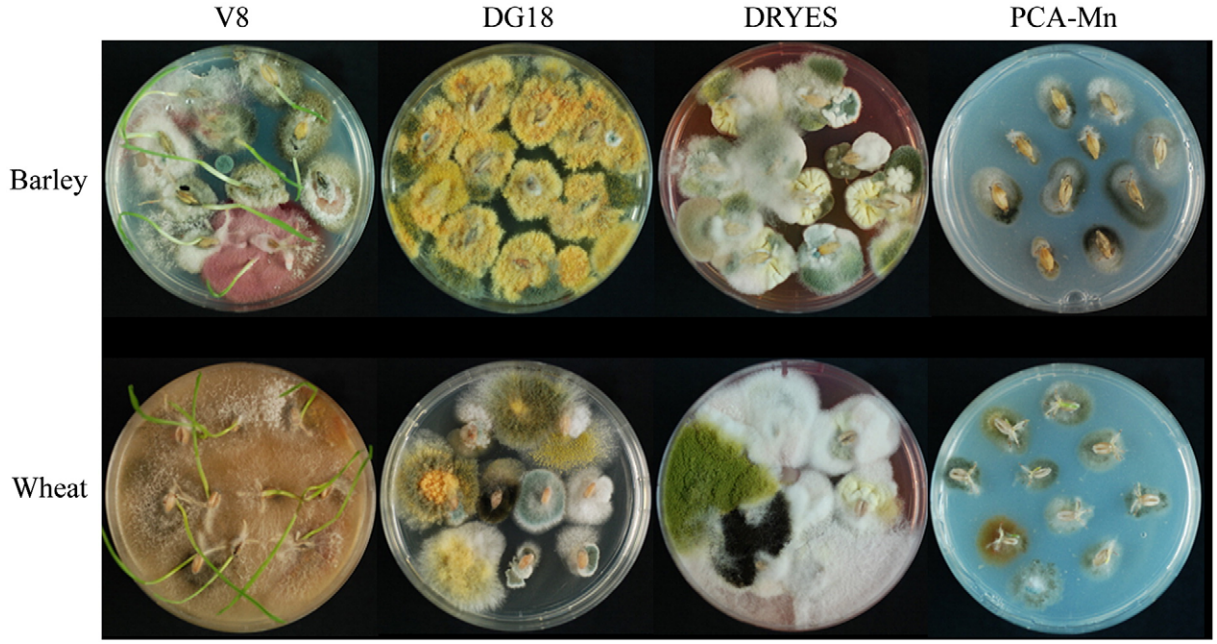

Fig. 3. Barley and wheat grains incubated on V8, DG 18, DRYES and PCA-Mn.

in this experiment. The results of the eight grain samples showed that *Aspergillus*, *Eurotium*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* were the predominant genera emerging on DG18, DRYES and V8 (Fig. 2). Some of the most frequently identified species were *Asp. flavus*, *Asp. niger*, *Eur. amstelodami*, *Eur. chevalieri*, *F. equiseti*, *F. tricinctum*, *F. verticillioides* and *Pen. chrysogenum*. None of these genera were observed on PCA-Mn, except for a *Rhizopus* sp. which emerged from one spelt grain and *F. verticillioides* which emerged from five spelt grains. One *Bipolaris* sp. emerged from 4% of the barley grains on PCA-Mn, which was comparable to DRYES where it emerged from 3% of the grains, indicating that this genus may not be inhibited by the manganese concentration used. *Alternaria infectoria* and *E. nigrum* were observed in the three cereal grain samples, spelt, barley and wheat. *Epicoccum nigrum* could, however, only be observed on PCA-Mn and not on DG18, DRYES or V8. *Alternaria infectoria* was observed to a lesser degree on DG18, DRYES and V8 compared to PCA-Mn, spelt (6% on V8 vs. 11% on PCA-Mn), wheat (1% on DG18 vs. 71% on PCA-Mn) and barley (6% on V8 vs. 96% on PCA-Mn). Representative plates with wheat and barley grains (Fig. 3) are shown to illustrate how *Alternaria* and *Epicoccum* are suppressed by competing fungi on DG18, DRYES and V8 and how they were able to emerge on PCA-Mn.

Aspergillus, *Fusarium*, *Mucor*, *Rhizopus* and *Trichoderma* were predominant, emerging from the two soil samples on DG18, DRYES and V8, of which only *F. oxysporum* were observed on PCA-Mn. One *Phoma eupyrena* and one *Paraconiothyrium minitan* emerged on PCA-Mn from the Danish soil sample, but were not observed on DG18, DRYES or V8. An unidentified black-spored species was isolated from the Ugandan soil sample with PCA-Mn. The isolate produced pigmented, globose spores and hairy pycnidia, but the ITS sequence failed to give a 100% match to a known species in GenBank. The best hit was an unknown leaf litter ascomycete (accession number AF502679), which was 99% (438/439 nucleotides) identical. The closest identified species was *Didymella bryoniae* (anamorph: *Phoma cucurbitacearum*; accession number AF297228), which was 88% identical.

4. Discussion

Alternaria, *Epicoccum* and *Phoma* are often suppressed by other faster growing fungi when the mycobiota of plant and soil samples are examined using standard media like DG18, DRYES and V8. This often hampers the detection and isolation of these three genera. This was also observed in this study as *E. nigrum* and *Phoma* spp. could only be isolated from potato carrot agar with manganese (PCA-Mn) and not from the other standard media. *Alternaria infectoria* was likewise observed in low numbers on DG18, DRYES or V8, but was the predominant species on PCA-Mn when present in the samples.

Potato carrot agar (PCA) was chosen as the basal medium because the three target genera, *Alternaria*, *Epicoccum* and *Phoma*, all sporulate well on this medium. The basal medium contains half the amounts of potato and carrot than a standard PCA, so that the limited food source in the substrate also became a stress factor for some genera. Furthermore, PCA was chosen as basal medium because it is the preferred medium for *Alternaria* identification (Simmons, 2007) and the sporulation patterns on PCA-Mn of the *Alternaria* species investigated resemble those on standard PCA. PCA-Mn can therefore also be used directly in numeration of *Alternaria* at the species-group level without the further isolation steps.

Manganese was chosen because it previously has been shown to have a greater inhibitory effect on Zygomycetes, *Aspergillus* and *Cladosporium* than on *Alternaria* (Schmitz et al., 1993). *Penicillium citrinum* and *A. alternata* were equally inhibited by manganese in the study by Schmitz et al. (1993). The *Penicillium* species investigated in this study were, however, not able to grow on PCA-Mn, which may be due to a combined effect of manganese and the potato and carrot food source. As observed in our study, *Fusarium* species were also tolerant to manganese in the study of Schmitz et al. (1993).

There seems to be a differentiation in manganese tolerance between *Fusarium* species. In the early development process we observed that *F. graminearum* had an intermediate tolerance (able to grow at 1% but not on 2% $MnCl_2 \cdot 4\ H_2O$ (w/v)) whereas *F. verticillioides* had a high tolerance (able to grow at 3% $MnCl_2 \cdot 4\ H_2O$ (w/v)). In our experiments with the various environmental samples we were able to isolate *F. equiseti*, *F. oxysporum*, *F. poae*, *F. tricinctum* and *F. verticillioides* with the three standard media, but only *F. oxysporum* and *F. verticillioides* could be isolated on PCA-Mn. Growth of additional species of *Alternaria* and *Phoma*, including *A. alternata*, *A. brassicicola*, *A. longipes*, *A. turkisafria* and *P. viridiae*, were tested in the early development stages, and they were all able to grow on PCA-Mn (data not shown). This indicates that manganese tolerance is very common

in these two genera. PCA-Mn can therefore most likely be used for isolation of all *Alternaria* and *Phoma* species, although further experiments are needed to verify this.

The concentration of $MnCl_2 \cdot 4\ H_2O$ can be increased at least up to 3% (w/v), if the medium is used solely for isolating *Phoma* spp. or *Epicoccum* sp. as these genera seem to be more tolerant to manganese than *Alternaria*. Increased manganese concentration will not eliminate growth of *F. verticillioides*, as this species appears to be as tolerant to manganese as *Phoma* and *E. nigrum* when the basal medium is PCA. There are several other factors, such as incubation temperature and manganese concentration which possibly can be optimized further in experiments focusing solely on one genus.

5. Conclusion

PCA-Mn was successfully developed to isolate these three genera from soil and plant samples, which contained other genera that prevailed on commonly used isolation media. PCA-Mn has several advantages compared to previously-developed semi-selective media for isolation of small-spored *Alternaria*. While the selectivity of other media is based on fungicides, either thiabendazole (Hong and Pryor 2004) or a combination of dichloran and rose bengal (Andrews and Pitt, 1986), PCA-Mn is based on easy available manganese. As PCA-Mn is based on PCA, the preferred medium for spore recognition in *Alternaria* (Simmons, 2007), PCA-Mn can make it easier to detect otherwise overlooked or suppressed *Alternaria* isolates in environmental samples.

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A polyphasic approach to the taxonomy of the *Alternaria infectoria* species–group

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ABSTRACT

Different taxa in the species–group of *Alternaria infectoria* (teleomorph *Lewia* spp.) are often isolated from various cereals including barley, maize and wheat grain, ornamental plants and skin lesions from animals and humans. In the present study we made a polyphasic characterization of 39 strains morphologically identifiable as belonging to the *A. infectoria* species–group together with 12 strains belonging to closely related species: *Alternaria malorum* (syn. *Cladosporium malorum*), *Chalastospora cetera* (syn. *Alternaria cetera*) and *Embellisia abundans*. Morphological examination separated the 51 strains in three groups based on conidial appearance and arrangement: the *A. infectoria* species–group, *E. abundans* and a group containing *C. cetera* and *A. malorum*. The metabolite analyses on three different media showed two clusters, one containing all 39 *A. infectoria* species–group strains and one containing 10 strains of *E. abundans*, *C. cetera* and *A. malorum*. One *E. abundans* strain and one *A. malorum* strain were not included due to insufficient metabolite production. The separation of the *A. infectoria* species–group from *E. abundans*, *C. cetera* and *A. malorum* resulted mainly from the ability to produce altertoxins and novae-zelandins. The metabolite analyses also showed that all 51 strains were able to produce infectopyrones. The metabolite profiles of *C. cetera* and *A. malorum* were very similar with several metabolites of unknown structure in common. This is the first time that *E. abundans*, *C. cetera* and *A. malorum* have been reported as producers of infectopyrones. Sequence analyses of the internal transcribed spacer region (ITS), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and translocation elongation factor 1α (*tef-1α*) showed two clades: one with the 39 strains from the *A. infectoria* species–group and one with the 12 strains of *E. abundans*, *C. cetera* and *A. malorum*. The polyphasic approach in this study suggests that *A. malorum* var. *polymorpha* and the eight *A. malorum* strains do not belong in *Alternaria*, but in *Chalastospora*, however, as several distinct species. Splits Tree alignment of *gpd* sequences of 38 strains belonging to the *A. infectoria* species–group indicates that only three strains showed signs of recombination, while the remaining strains appeared to be clonal. Long term incubation at 7 °C in the dark showed that 12 out of 33 tested strains from the *A. infectoria* species–group were able to produce proascomata in axenic culture, but with no mature ascospores after 6 months. These findings suggest that *Lewia*/*A. infectoria* species–group must, at least in part, be homothallic. The results presented in this study show that ITS, *tef-1α* and *gpd* do not reflect ecology, secondary metabolism or morphology of the *A. infectoria* species–group and that molecular cladification and phylogeny cannot predict pathogenicity, host specificity or mycotoxin production.

1. Introduction

The *Alternaria infectoria* EG Simmons species–group *sensu* Simmons (Simmons and Roberts, 1993; Simmons, 2007) comprises more than 30 named anamorph taxa, among which *Alternaria arbusti* EG Simmons, *Alternaria ethzedia* EG Simmons, *A. infectoria*, *Alternaria intercepta* EG Simmons, *Alternaria metachromatica* EG Simmons, *Alternaria oregonensis* EG Simmons, *Alternaria photistica* EG Simmons, *Alternaria triticimaculans* EG Simmons, *Alternaria triticina*, *Alternaria viburni* EG Simmons are some (Simmons, 2007). It is the only group in *Alternaria* where some members have a teleomorph state, *Lewia* ME Barr and EG Simmons (Simmons, 1986). Morphologically, the *A. infectoria* species–group differs from other *Alternaria* species–groups in the three-dimensional sporulation pattern (Simmons and Roberts, 1993). Characteristic for the *A. infectoria* species–group is the production of small conidia (up to 70 μm in length) in branched chains with long, geniculate multilocus secondary conidiophores (up to 120 μm) between conidia (Simmons, 2007).

Chemically, the *A. infectoria* species–group is very different from other *Alternaria* species, producing metabolites that are not found

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in other species–groups (Andersen and Thrane, 1996). None of the taxa in the *A. infectoria* species–group has ever been shown to produce alternariols or tenuazonic acid, which are common in other small-spored *Alternaria* species (Andersen et al., 2002) or altersolanols, common in some large-spored *Alternaria* (Andersen et al., 2008). On the other hand, taxa in the *A. infectoria* species–group produce infectopyrones and novae-zelandins (Christensen et al., 2005), which have never been detected in other *Alternaria* species–groups. However, infectopyrones have been found in other genera, such as *Phoma* Sacc., *Stemphylium* Wallr. and *Ulocladium* Preuss (Pedras and Chumala, 2005; Christensen et al., 2005; Andersen and Hollensted, 2008, respectively).

Molecularly, taxa in the *A. infectoria* species–group have been analyzed at the sub-genus level using the ribosomal internal transcribed spacer region (ITS), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and translocation elongation factor 1α (*tef-1α*) sequences and the results showed that the *A. infectoria* species–group constitutes a quite distinct clade (de Hoog and Horré, 2002; Pryor and Bigelow, 2003). Another ITS sequence analysis creating an unrooted radial tree based on maximum likelihood calculation showed that *Alternaria malorum* (Ruehle) U. Braun, Crous and Dugan (*Cladosporium malorum* Ruehle), and the *A. infectoria* species–group comprised a single clade (Braun et al., 2003). An inquiry on *Alternaria* in the CBS database of ITS sequences showed that a strain of *Chalastospora cetera* (EG Simmons) EG Simmons (*Alternaria cetera* EG Simmons) and three strains of *Embellisia abundans* EG Simmons also clustered in the same clade as *A. malorum* and the *A. infectoria* species–group (unpublished results).

Many taxa in the *A. infectoria* species–group are associated with various species in the grass family (*Poaceae* L.). They have been reported from stems, straw, leaves and grains of oat, barley, wheat and rye (Simmons, 1986; Andersen et al., 2002; Dugan and Peever, 2002; Perelló et al., 2008) and are also known to occur on maize (unpublished results). *A. triticina* Prasada and Prabhu, a known plant pathogenic species in the *A. infectoria* species–group, was first reported on wheat in India (Prasada and Prabhu, 1962) and later on the same host plant in Argentina (Perelló and Sisterna, 2006) and in Iran (Simmons, 2007). Other species isolated from discrete lesions of non-poaceae host plants, such as *A. viburni* and *A. ethzedia*, are presumed to have various degrees of pathogenicity (Simmons, personal commun.). In the last decade, taxa in the *A. infectoria* species–group have increasingly been isolated from human cutaneous infections, especially from immuno-compromised patients (de Hoog and Horré, 2002; Dubois et al., 2005; unpublished results).

The objective of this work was to prove the hypothesis that taxa in *Lewia*/*A. infectoria* species–group are sexual fungi and that molecular sequence analysis and metabolite profiling will yield a number of clades and clusters that will correspond to the number of morphological species in the group. Previous research on other genera has shown that sequence analyses reveal cryptic molecular species (Taylor et al., 2000; O'Donnell et al., 2004). Studies on *Penicillium* and large-spored *Alternaria* have shown that results from metabolite profiling correlates with the morphological species concept (Andersen et al., 2008; Frisvad and Samson, 2004). Other research on *Aspergillus* and *Stachybotrys* has shown that molecular sequence analyses correlated with the morphological species concept and metabolite profiling (Samson et al., 2007; Andersen et al., 2003). Furthermore, controversy over whether the *A. infectoria* species–group comprises many species based on morphological differences or consists of only one species based on molecular sequence analysis has arisen. Therefore, this polyphasic study of the *A. infectoria* species–group, including molecular sequence analysis, metabolite profiling and mating tests, was set up. The study also included *A. malorum,* since this species had been reported to belong to the *A. infectoria* species–group (Braun et al., 2003), together with *C. cetera* and *E. abundans*. One aim was to compare chemical and molecular findings with the current morphological classification and examine which factors correlate and which could resolve and segregate *Alternaria* from *Chalastospora* and *Embellisia*. Another aim was to test if other isolates than the human opportunists were able to grow at 37 °C.

2. Materials and methods

2.1. Fungal strains

Fifty-one fungal strains belonging to the *A. infectoria* species–group (39), *A. malorum* (9), *C. cetera* (1), and *E. abundans* (2) were used. Identity, species–group affiliation, identification number, host plant, and geographic origin of all strains are given in Table 1. Strains are available from CBS collection, Fungal Biodiversity Centre, The Netherlands, and IBT collection, Department of Systems Biology, DTU, Denmark.

2.2. Morphological examination

For morphological examination and DNA analysis, each strain was inoculated in three points onto a PCA plate (potato carrot agar; Simmons, 2007) and incubated under standardized conditions (Andersen et al., 2005). In brief: after inoculation the unsealed plates were incubated in one layer for 7 days at 23 °C under an alternating light/dark cycle consisting of 8 h of cool-white fluorescent daylight (tubes: TLD, 36 W/95, Philips, Denmark) and 16 h darkness. Slides for microscopy were made after 7 days using transparent tape preparations (Butler and Mann, 1959) mounted in lactophenol. All unidentified strains were compared with type cultures and descriptions according to Simmons (2007).

For ascoma production under laboratory conditions, strains were transferred to another PCA plate that had been equipped with autoclaved wooden toothpicks to encourage the production of ascomata. In the first trial, each plate was divided into three sectors with three toothpicks. Three different strains were inoculated on the same plate, one in each sector. In the second trial, each plate was divided into two sectors with one toothpick. The same strain was inoculated in both sectors on the same plate. The plates were first incubated as ordinary PCA plates under alternating light at 23 °C for 2 weeks. Then the plates were sealed with para film™, placed up-side-down, and incubated at 7 °C in the dark for 6 months.

For the ability to grow at high temperature, strains were transferred (three points) to PDA plates (potato dextrose agar; Difco, 213400). Plates were allowed to stand at room temperature for one day to ensure that all strains were viable and growing. The edge of the colonies was marked before incubation. After 12 days at 37 °C the colony edges were marked again and the plates were allowed to stand for two days at room temperature. Strains that had resumed their original growth characteristics were recorded as positive.

2.3. Metabolite extraction

For metabolite analyses, each strain was transferred (three points) onto a DRYES plate (dichloran Rose Bengal yeast extract sucrose agar; Frisvad, 1983), a DG18 plate (dichloran 18% glycerol agar [31.5 g/l dichloran glycerol agar base (OXOID, CM0729); 220 ml/l anhydrous glycerol (JT Baker, 7044); 10 mg/l $ZnSO_4 \cdot 7H_2O$ (Merck, 8883); 5 mg/l $CuSO_4 \cdot 5H_2O$ (Riedel-de Haën, 12849); 50 mg/l chloramphenicol (Sigma, C-0378); 50 mg/l chlortetracycline hydrochloride (Sigma, C-4881)]), and a PDA + DN plate (potato dextrose agar [39.0 g/l potato dextrose agar (Difco, 213400),

Table 1
Host, origin and species–group affiliation of the *Alternaria*, *Chalastospora* and *Embellisia* strains and type cultures (T) used in this study.

#[a]	Genus species	species–group[b]	Host	Origin	CBS #	BA #	EGS #
1	*A. infectoria* sp.–grp	inf. sp.–grp.	*Hordeum*, grain	DK, Jutland	120147	704	
2	*A. triticina* T	inf. sp.–grp.	*Triticum*	India	763.84	1207	17–061
3	*A. infectoria* T	inf. sp.–grp.	*Triticum*	UK	112250	1209	27–193
4	*A. oregonensis* T	inf. sp.–grp.	*Triticum*	USA, OR	542.94	1210	29–194
5	*A. photistica* T	inf. sp.–grp.	*Digitalis*	UK	212.86	1211	35–172
6	*A. ethzedia* T	inf. sp.–grp.	*Brassica*	Switzerland	197.86	1215	37–143
7	*A. metachromatica* T	inf. sp.–grp.	*Triticum*	Australia	553.94	1218	38–132
8	*A. triticimaculans* T	inf. sp.–grp.	*Triticum*	Argentina	578.94	1221	41–050
9	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*	Italy		1228	42–086
10	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*	NZ		1234	43–070
11	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*	NZ		1239	43–160
12	*A. intercepta* T	inf. sp.–grp.	*Viburnum*	Europe	119406	1258	49–137
13	*A. viburni* T	inf. sp.–grp.	*Viburnum*	Europe	119407	1259	49–147
14	*A. arbusti* T	inf. sp.–grp.	*Pyrus*	USA, CA	596.93	1263	91–136
15	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Triticum*, grain	DK	120148	1286	
16	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Triticum*, grain	DK	120149	1287	
17	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*, grain	DK	120150	1294	
18	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*, grain	DK	120151	1312	
19	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*, grain	DK	120152	1315	
20	*C. cetera* T		*Elymus*	Australia	110898	1746	41–072
21	*A. malorum*		Soil	Syria	173.80	1747	
22	*A. malorum*		–	USA	148.66	1748	
23	*A. malorum*		–	NZ	114810	1749	
24	*A. malorum*		–	NZ	114809	1750	
25	*A. malorum*		Soil	Lebanon	900.87	1751	
26	*A. malorum* var. *polymorpha*		*Vitis*	USA, WA	112048	1752	
27	*A. malorum*		*Triticum*, straw	SA, Cape Prov.	266.75	1753	
28	*A. malorum*		*Triticum*, grain	USA, OR	216.65	1754	
29	*A. malorum*		*Gossypium*, seed	Turkey	540.75	1755	
30	*E. abundans*		*Dianthus*, seed	UK	535.83	1757	34–063
31	*E. abundans* T		*Fragaria*	NZ	534.83	1758	29–159
32	*A. infectoria* sp.–grp.	inf. sp.–grp.	–	UK	160.79	1759	
33	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, Jutland		1760	
34	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, Jutland		1761	
35	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, N Jutland		1762	
36	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, N Jutland		1763	
37	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, NW Jutland		1764	
38	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, Jutland		1765	
39	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, S Jutland		1766	
40	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, Jutland		JLS08	
41	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, Jutland		JLS09	
43	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, N Jutland		JLS32	
44	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, E Jutland		JLS33	
45	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Homo*, skin lesion	Germany	102692		
46	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Hordeum*	UK	116001		
47	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Paeonia*	NL	106.52		
48	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Avena*, straw	–	308.53		
49	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Homo*, skin lesion	Italy	109785		
50	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Homo*, skin lesion	NL	110803		
51	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Homo*, skin lesion	Austria	110804		
52	*A. infectoria* sp.–grp.	inf. sp.–grp.	*Zea*, fresh silage	DK, N Jutland		JLS38	

[a] Analysis number in this study. #42 is not included.
[b] Member of the *A. infectoria* species–group based on morphology.

10 mg/l $ZnSO_4 \cdot 7H_2O$ (Merck, 8883) and 5 mg/l $CuSO_4 \cdot 5H_2O$ (Riedel-de Haën, 12849)] with additional 30 g/l dextrose (BHD, 10117) and additional 3 g/l $NaNO_3$ (Merck, A855537 811). After inoculation, the DRYES, DG18, PDA + DN plates were packed in perforated plastic bags and incubated in darkness at 25 °C prior to extraction.

Metabolites were extracted separately from 14-day-old DRYES, DG18 and PDA + DN cultures. The extractions were done using a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al., 2005). In brief: three 6-mm agar plugs were cut from the center of the three colonies and the nine plugs were placed in a 2-ml vial. Plugs were extracted with 1.0 ml ethyl acetate containing 1% formic acid (v/v) and ultrasonicated for 60 min. Extracts were transferred to clean 2-ml vials, evaporated to dryness, re-dissolved ultrasonically in 400 μl methanol, and filtered through 0.45-μm PFTE filters (National Scientific Company, Rockwood, TN, USA) into clean 2-ml vials prior to HPLC analysis. Experiments were repeated once on DRYES a year after with all strains and for strains with low metabolite production a third time on DRYES a month later using 18 plugs.

2.4. HPLC-UV–VIS analyses

Metabolite profiling was performed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped a diode array detector collecting two ultraviolet–visible (UV–VIS) spectra per sec from 200 to 600 nm. Samples of 3.0 μl were separations on a 2 × 100 mm Luna 3 μm C18(2) (Phenomenex, Torrance, CA, USA) at 40 °C using a linear water–acetonitrile gradient and a flow of 0.4 ml/min. The gradient started at 15% acetonitrile, reached 100% in 20 min and was held for 5 min. Both eluents contained 50 ppm trifluoro acetic acid. A homologous series of alkylphenones

was analyzed as external retention time references and used to calculate a bracketed retention index (RI) for each detected peak (Frisvad and Thrane, 1987). In this way each metabolite could be identified by its RI value and its UV–VIS spectrum and be recognized in other extracts.

2.5. HPLC-TOF-MS analyses

Metabolite identification was done on the DRYES extracts using on a similar HPLC-DAD system as described above with minor changes: the column was 50 mm long, flow was 0.3 ml/min, and the eluents were buffered with 20 mM formic acid (Nielsen et al., 2005). High resolution MS detection was done on a Time Of Flight mass spectrometer (Water-Micromass, Manchester, UK) scanning m/z 60–900 and m/z 100–2000 in two separate scan functions at different skimmer settings (Nielsen et al., 2005). All samples were analyzed in both positive (ESI^+) and negative electrospray (ESI^-). Extracts were also analyzed using a gradient system optimized for *A. infectoria* metabolites, using a 2 mm × 100 mm 3 μm Gemini Phenyl column (Phenomenex, Torrance, CA, USA) and a gradient starting at 10% acetonitrile, reaching 47% in 17 min and then 100% in 3 min and was held for 4 min. All samples were analyzed in ESI^+ and ESI^-. Metabolite standards (Nielsen and Smedsgaard, 2003) of 4*Z*-infectopyrone, AAL-toxins TA and TB, AK toxin I, altenuene, altenusin, alternariol, alternariol monomethylether, altersolanol A, altertoxin I, macrosporin, maculosin, tentoxin and tenuazonic acid were co-analyzed for verification.

2.6. Data treatment of metabolite profiles

Metabolite profile data from HPLC-UV–VIS were first treated with the Chemical Image Analysis (CIA) program using an algorithm described by Hansen (2003) as stated by Andersen et al. (2008). In brief: the raw HPLC data files, which are quantitative 2-D matrices (x-axis: time, y-axis: wave length, value in matrix: UV–VIS absorbance), were transferred from the HPLC to a standard PC and analyzed by an in-house written chemical image analysis (CIA) program (Hansen, 2003). No manipulations or peak selections were made before processing. Each HPLC file was processed first by a $\log_{10}$ scaling (to account for concentration differences among extracts), then a baseline correction and finally an alignment (to account for drift in baseline and retention time among identical metabolites in different runs) (Hansen, 2003). Each HPLC file was then compared to the other 50 HPLC files, pair-wise, using an algorithm described by Hansen (2003) giving a similarity value for each pair, which was entered into a new matrix. The resulting 51 × 51 similarity matrix was then used to calculate a dendrogram using WARD clustering method.

Based on the result of the CIA, a binary matrix was made manually by scoring each metabolite from four printed HPLC chromatograms as present or absent (137 metabolites for the 51 fungal strains) and subjected to multivariate statistics using Unscrambler version 9.2 (CAMO ASA, Oslo, Norway). The matrix was analyzed using Partial Least Squares Regression (PLS-R) and Principal Component Analysis (PCA). A reduced matrix (124 metabolites and 49 strains) was subjected to cluster analysis using NTSYS-pc version 2.11 N (Exeter Software, Setauket, NY, USA) without standardization using Yule as correlation coefficient and Unweighted Pair-Group Method using arithmetic Averages (UPGMA) as clustering method. The matrix was also analyzed by simple matching and Jaccard similarity coefficients in NTSYS.

For metabolite identification and peak comparison, 12–20 of the largest peaks in the data files from HPLC-TOF-MS were inspected in UV–VIS, ESI^+, ESI^- modes were compared to peaks registered in the Quanlynx 4.1 software (Water-Micromass) using a $\pm m/z$ 0.02 and a retention time limit of ±0.3 min. If possible, a qualifier ion was used for confirmation. Peaks of the metabolite standards mentioned above were also inserted along with predicted ions calculated from the masses of known *Alternaria* metabolites. In the latter case a ±3 min window was used, with the retention time predicted using the log *D* of the compound (calculated using ACD v.10, Advanced Chemical development Inc., Toronto, Canada), which was correlated to retention time of 50 representative secondary metabolites. All extracts were then analyzed by the Quanlynx software. For metabolite identification, each peak was matched against an internal reference standard database (~800 compounds) as well as tentatively identified by searching the accurate mass in the 34,392 compounds in Antibase 2008 (Laatsch, 2008), comparing UV–VIS data, fragmentations, ionization efficiency in ESI^- versus ESI^+ and the retention time to information in the databases.

2.7. DNA extraction, PCR amplification and sequencing

For DNA analysis, each strain was inoculated in three points onto a PCA plate and incubated as mentioned above. DNA was extracted from 7-day-old PCA cultures with UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer's protocol and stored at −20 °C. Amplifications of the three target genes were performed with the following primer combinations: ITS: V9G (de Hoog and Gerrits van den Ende, 1998) and ITS4 (White et al., 1990): *gpd*: gpd1 and gpd2 (Berbee et al., 1999), *tef-1α*: EF1–645F: (TCG TCG TYA TCG GMC ACG TCG A) and EF1–1190R (TAC CAG TGA TCA TGT TCT TGA TGA). EF1–645F and EF1–1190R were designed by aligning sequences of *Gibberella zeae* (XM388987), *N. crassa* (D45837), *Aspergillus fumigatus* (XM745295) and *Ustilago maydis* (XM751978) from GenBank. The numbers of the primers refer to the nucleotide position in *N. crassa* (D45837) at the 3′-end as done previously (Carbone and Kohn, 1999). ITS and *gpd* PCR reactions were performed in 12.5 μl volumes containing 0.5 μl DNA, 1 × NH_4^+-buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl (pH 8.8 at 25 °C), 0.1% Tween-20] (Bioline, London, UK), 1 mM $MgCl_2$, 0.04 mM dNTPs, 0.2 pmol of each primer and 0.5 U BIOTAQ™ DNA Polymerase (Bioline, London, UK). *Tef-1α* PCR reactions were performed with 0.8 pmol of each primer and 2.5 mM $MgCl_2$ and with the same concentrations of the remaining ingredients as for ITS and *gpd*. All PCR amplifications were performed after the same scheme with an initial denaturation at 94 °C for 5 min followed by 40 amplification cycles of 94 °C for 30 s, annealing for 30 s and 72 °C for 1:20 min, and a final extension at 72 °C for 7 min. Annealing temperatures were: 48 °C (ITS), 52 °C (*tef-1α*) and 59 °C (*gpd*). Amplicons were run on 1% agarose gels and visualised with UV after ethidium bromide staining. Sequence reactions were performed with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). All sequence-PCR reactions were performed with the same protocol: 94 °C for 1 min, followed by 30 cycles of 94 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. DNA was purified with Sephadex® G-50 (Pharmacia-Amersham) and sequenced. All sequences determined in this study have been submitted to GenBank and accession numbers are listed in Table 2.

2.8. Data treatment of molecular sequences

Sequence electrophorograms of forward and backward runs were combined, analyzed, edited using DnaStar SeqMan II (LaserGene). Sequence data were saved and aligned with BioNumerics (Applied Maths, Kortrijk, Belgium). The alignments of *tef-1α*, ITS and *gpd* were concatenated into one alignment to construct a phylogenetic tree. The program RaxML (http://www.phylo.org/portal/Home.do) was used to create the best tree using maximum likelihood and to calculate bootstrap values (Stamatakis et al., 2008). The same program and conditions were used to create individual trees of all three markers used in this study. Genetic diversity of

Table 2
Ascoma production on PCA and growth at 37 °C on PDA together with *gpd* haplotypes and GenBank accession numbers.

#[a]	Genus (species/group)	Ascoma production		Growth at 37 °C	*gpd*	ITS	*gpd*	*tef-1α*
		1. Trial	2. Trial		Haplotype			
1	*Alternaria infectoria* sp.–grp.	+	+	+	1	FJ214885	FJ214836	FJ214932
2	*A. triticina* T (inf–grp)	–	–	+	3	AY762948	FJ214846	FJ214942
3	*A. infectoria* T (inf–grp)	+	–	–	2	FJ214897	FJ214850	FJ214946
4	*A. oregonensis* T (inf–grp)	+	–	+	4	AY762947	FJ214849	FJ214945
5	*A. photistica* T (inf–grp)	–	+	–	nt	FJ214900	FJ214854	FJ214950
6	*A. ethzedia* T (inf–grp)	+	+	+	5	AY278833	FJ214855	FJ214951
7	*A. metachromatica* T (inf–grp)	–	–	–	6	AY762946	FJ214835	FJ214931
8	*A. triticimaculans* T (inf–grp)	–	–	–	7	AY762949	FJ214834	FJ214930
9	*A. infectoria* sp.–grp.	+	–	+	21	FJ214890	FJ214841	FJ214937
10	*A. infectoria* sp.–grp.	+	–	–	22	FJ214886	FJ214837	FJ214933
11	*A. infectoria* sp.–grp.	+	–	–	22	FJ214859	FJ214808	FJ214904
12	*A. intercepta* T (inf–grp)	+	–	+	8	FJ214882	FJ214831	FJ214927
13	*A. viburni* T (inf–grp)	+	–	+	9	FJ214876	FJ214825	FJ214921
14	*A. arbusti* T (inf–grp)	–	–	+	10	FJ214857	FJ214806	FJ214902
15	*A. infectoria* sp.–grp.	+	+	+	11	FJ214887	FJ214838	FJ214934
16	*A. infectoria* sp.–grp.	+	+	–	12	FJ214863	FJ214812	FJ214908
17	*A. infectoria* sp.–grp.	+	+	–	2	FJ214865	FJ214814	FJ214910
18	*A. infectoria* sp.–grp.	+	–	+	13	FJ214866	FJ214815	FJ214911
19	*A. infectoria* sp.–grp.	+	+	–	9	FJ214868	FJ214817	FJ214913
20	*Chalastospora cetera* T	nt[b]	–	–	nt	FJ214864	FJ214813	FJ214909
21	*A. malorum*	nt	–	+	nt	FJ214888	FJ214839	FJ214935
22	*A. malorum*	nt	–	+	nt	FJ214870	FJ214819	FJ214915
23	*A. malorum*	nt	+	+	nt	FJ214894	FJ214845	FJ214941
24	*A. malorum*	nt	+	+	nt	FJ214861	FJ214810	FJ214906
25	*A. malorum*	nt	–	+	nt	FJ214860	FJ214809	FJ214905
26	*A. malorum* var. *polymorpha*	nt	–	+	nt	FJ214883	FJ214832	FJ214928
27	*A. malorum*	nt	+	+	nt	FJ214884	FJ214833	FJ214929
28	*A. malorum*	nt	–	+	nt	FJ214895	FJ214847	FJ214943
29	*A. malorum*	nt	–	+	nt	FJ214896	FJ214848	FJ214944
30	*Embellisia abundans*	nt	–	–	nt	FJ214898	FJ214851	FJ214947
31	*E. abundans* T	nt	–	–	nt	AB120848	FJ214852	FJ214948
32	*A. infectoria* sp.–grp.	nt	–	–	6	FJ214872	FJ214821	FJ214917
33	*A. infectoria* sp.–grp.	nt	–	+	23	FJ214873	FJ214822	FJ214918
34	*A. infectoria* sp.–grp.	nt	+	+	24	FJ214862	FJ214811	FJ214907
35	*A. infectoria* sp.–grp.	nt	+	+	15	FJ214880	FJ214829	FJ214925
36	*A. infectoria* sp.–grp.	nt	–	+	15	FJ214867	FJ214816	FJ214912
37	*A. infectoria* sp.–grp.	nt	+	+	15	FJ214871	FJ214820	FJ214916
38	*A. infectoria* sp.–grp.	nt	+	–	9	FJ214875	FJ214824	FJ214920
39	*A. infectoria* sp.–grp.	nt	–	+	15	FJ214881	FJ214830	FJ214926
40	*A. infectoria* sp.–grp.	nt	–	+	19	FJ214877	FJ214826	FJ214922
41	*A. infectoria* sp.–grp.	nt	–	+	9	FJ214879	FJ214828	FJ214924
43	*A. infectoria* sp.–grp.	nt	nt	nt	2	FJ214892	FJ214843	FJ214939
44	*A. infectoria* sp.–grp.	nt	–	+	20	FJ214878	FJ214827	FJ214923
45	*A. infectoria* sp.–grp.	nt	–	–	14	FJ214856	FJ214805	FJ214901
46	*A. infectoria* sp.–grp.	nt	–	+	15	FJ214899	FJ214853	FJ214949
47	*A. infectoria* sp.–grp.	nt	+	–	6	FJ214858	FJ214807	FJ214903
48	*A. infectoria* sp.–grp.	nt	nt	nt	16	FJ214889	FJ214840	FJ214936
49	*A. infectoria* sp.–grp.	nt	nt	nt	17	FJ214869	FJ214818	FJ214914
50	*A. infectoria* sp.–grp.	nt	nt	nt	15	FJ214874	FJ214823	FJ214919
51	*A. infectoria* sp.–grp.	nt	nt	nt	18	FJ214891	FJ214842	FJ214938
52	*A. infectoria* sp.–grp.	nt	nt	nt	15	FJ214893	FJ214844	FJ214940

[a] Analysis number in this study. #42 is not included.
[b] Not tested.

aligned sequences and the standardized Index of Association (I_A^s) was calculated with LIAN 3.5 (Haubold and Hudson, 2000) by combining clustering information of the individual trees. The phylogenetic network was created with SplitsTree v4.8. The network structure was based on the neighbornet algorithm with a threshold set to 10^{-4} and applying the LogDet transformation. LogDet is a distance transformation that corrects for biases in the base composition (Wägele and Mayer, 2007). The *phi*-test incorporated in the SplitsTree software (Huson and Bryant, 2006) was used to test signals of recombination ($p < 0.05$, significant evidence of recombination). The test is proven to be a robust calculation and no previous knowledge about population history, recombination rate, mutation rate and rate heterogeneity across sites (Bruen et al., 2006) is necessary. Although large splits in networks do not necessarily imply recombination, split decomposition networks in conjunction with the *phi*-test can easily detect which sequences in a given data set contribute the most to the recombination signal (Salemi et al., 2008). The *phi*-test is repeated after possible recombinants are deleted from the alignment until $p > 0.05$ (no evidence of recombination). DnaSP v4.5 (Rozas and Rozas, 1995) was used to find the different haplotypes in the *gpd* alignment. Gaps and missing data were not considered during calculation.

3. Results

3.1. Morphology

Examination of the sporulation patterns on PCA after seven days of growth in alternating light showed three different and very distinct morphologies as shown in Fig. 1. *A. malorum*, *A. malorum* var.

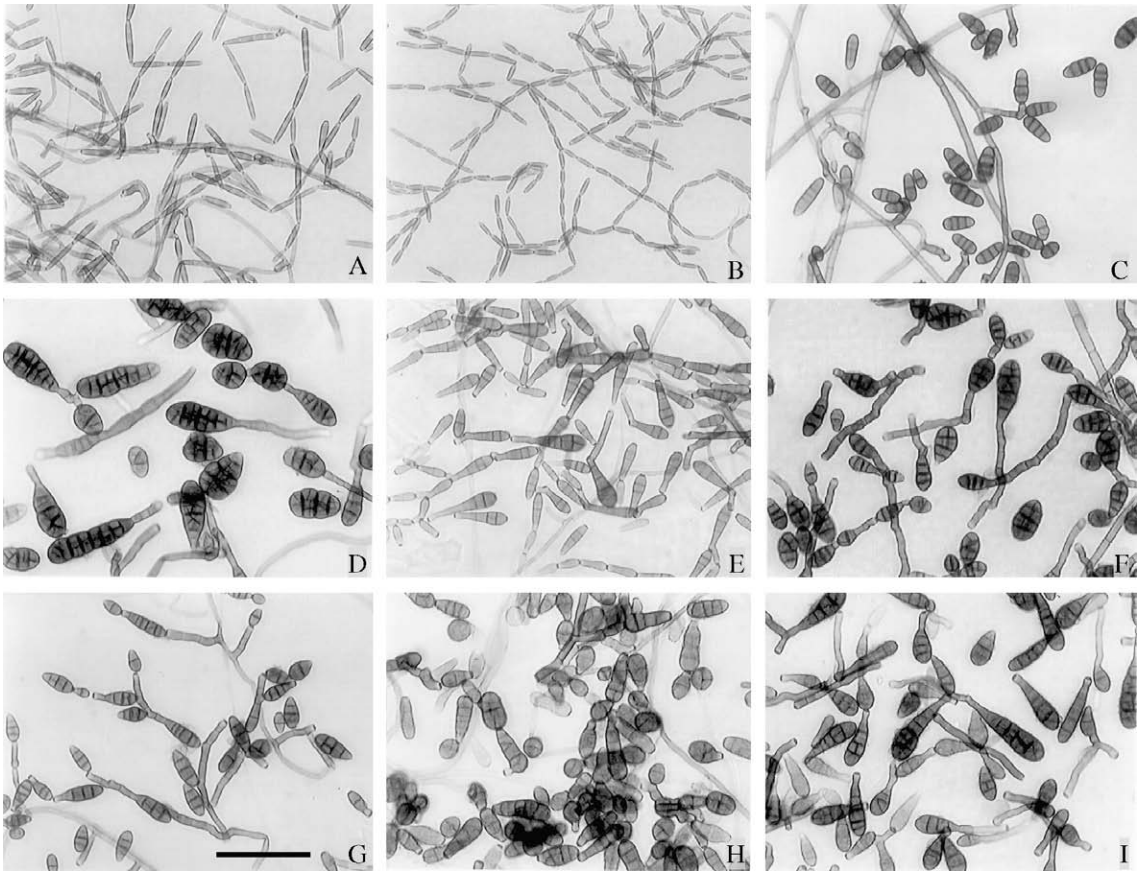

Fig. 1. Morphology plates. (A): *Chalastospora cetera* (#20), (B): *Alternaria malorum* (#25), (C): *Embellisia abundans* (#31) and six strains belonging to the *A. infectoria* species–group: (D): *A. oregonensis* (#04), (E): *A. infectoria* (#03), (F): *A. arbusti* (#14) and (G–I): three *A. infectoria* species–group strains (#32, #44 and #17, respectively). Bar in *G* = 50 μm.

polymorpha and *C. cetera* all had cylindrical didymo- and phragmoconidia produced in very long branching chains (Fig. 1A and B). Both strains of *E. abundans* had solitary, ovoid phragmoconidia (Fig. 1C), while all strains of the *A. infectoria* species–group shared the same morphology: ovoid, obpyriform or obclavate phragmo- and dictyoconidia with secondary conidiophores of varying length produced in branched chains (Fig. 1D–I). Type cultures fitted the descriptions, except *A. arbusti* (#14), which, under the growth conditions in this study, showed a more branched three-dimensional structure than the original description depicted (Simmons, 2007). Conidial sizes, shapes, ornamentation, color etc. varied greatly between strains, whereas conidial appearance was quite consistent within a strain: e.g. conidia of *A. infectoria* (#03) were smooth, narrow-obpyriform and sallow colored (Fig. 1E), while conidia of *A. infectoria* species–group (#44) were narrow-obpyriform to broad-ovoid, finely rough and bronze colored (Fig. 1H). None of the strains morphologically identified as belonging to the *A. infectoria* species–group could be assigned to any of the type cultures and no two strains, except *A. infectoria* species–group strains (#10 and #11), could be classified as belonging to the same taxon.

Table 2 shows the production of proascomata (ascomata without mature ascospores) after 6 months of incubation on PCA at 7 °C. In the first trial, where three strains were inoculated on the same PCA plate, no mating between strains was observed. Proascomata (Fig. 2) were formed in the center of each colony and in areas in the agar furthest away from the two other colonies and there was a clear demarcation line between colonies. In the second trial, where the same strain had been inoculated twice on the same plate, proascomata were again formed at the center and furthest away from the other colony. Fourteen out of nineteen *A. infectoria* species–group strains produced proascomata in the first trial, and only seven of these in the second trial. Additional 14 strains were tested in the second trial, out of which five produced proascomata. In the second trial, neither *C. cetera* nor *E. abundans* produced proascomata, but three out of the nine *A. malorum* did. In contrast to the strains of the *A. infectoria* species–group, the three *A. malorum* strains produced their proascomata on the toothpicks and not in the agar. When the experiment was terminated after 6 months, none of the proascomata had yielded any matured ascospores.

3.2. Growth on different media

The result of the experiment on PDA at 37 °C given in Table 2 showed that 28 out of the 45 tested strains were able to grow at this temperature. Unfortunately, it was not possible to test all 51 strains in the set, since six strains were not viable after the first chemical experiments. Table 2 shows that all nine *A. malorum* strains were able to grow at 37 °C, but not *C. cetera*. During the incubation period, the colonies of *A. malorum* became dark brown and the mycelium thinner and more thread-like. Of the 33 tested strains of *A. infectoria* species–group, 20 were able to grow at 37 °C. The colonies lost their aerial mycelium, became waxy in their growth, and only produced filamentous mycelium after they were taken out of the 37 °C incubator. The only viable strain of *A. infectoria* species–group from a human infection (#45) was not able to grow on PDA at 37 °C.

Examination of the colony appearance on DRYES incubated at 25 °C showed that most of the 51 strains produced hairy to granu-

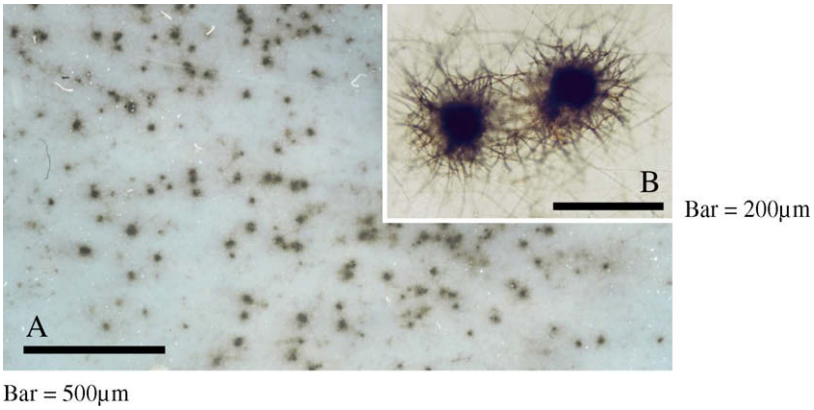

Fig. 2. Proascomata in 3-week-old PCA cultures. (A): *Alternaria infectoria* species–group #19 (bar = 500 µm) and (B): *A. infectoria* species–group #16 (bar = 200 µm).

lar mycelium, whitish to grayish in color. Some of the *A. infectoria* species–group strains produced only wet, yeast-like colonies on DRYES, especially the strains isolated from human lesions, which, however, did not affect their metabolite production. Filamentous mycelium was produced when the strains were transferred to PDA or PCA. Sporulation of the *A. infectoria* species–group was only seen on PCA and sometimes scarification and prolonged incubation were needed, while *A. malorum, C. cetera* and *E. abundans* sporulated well on PCA as well as on PDA + DN and PDA. DRYES and DG18 did not accommodate any sporulation. As a curiosity, it should be mentioned that *A. metachromatica* (#07) on PDA + DN produced an extracellular pigment that turned the reverse of the plate dark blue.

3.3. Chemical classification

The chemical HPLC-UV–VIS analyses of the three growth media, DRYES, DG18 and PDA + DN, showed that the metabolite production, qualitative as well as quantitative, was greatest on DRYES and that metabolite production seemed to be inhibited on PDA + DN. Fig. 3 shows six selected HPLC chromatograms made from the DRYES extracts.

Automated and unbiased chemical image analyses (CIA) of the HPLC-UV–VIS files were made with extracts from all three growth media to aid the selection of species-specific metabolites (dendrograms not shown). All three dendrograms gave the same grouping, but the one made with extracts from DRYES gave the most detailed dendrogram. It showed that the 51 strains grouped in four major clusters. One cluster contained all eight *A. malorum* strains, the *C. cetera* (#20) strain, and the type culture of *E. abundans* (#31). A second cluster contained 13 *A. infectoria* species–group strains, including the type cultures of *A. infectoria* (#03), *A. ethzedia* (#06), and *A. metachromatica* (#07), together with *E. abundans* (#30) and *A. malorum* var. *polymorpha* (#26). A third cluster contained 10 *A. infectoria* species–group strains, including the type cultures of *A. intercepta* (#12) and *A. viburni* (#13), while the last cluster contained 12 *A. infectoria* species–group strains, including type cultures of *A. photistica* (#05), *A. arbusti* (#14), *A. oregonensis* (#04), *A. triticina* (#02) and *A. triticimaculans* (#08). Visual examination of the HPLC chromatograms of *E. abundans* (#30) and *A. malorum* var. *polymorpha* (#26) showed very few peaks, meaning a very low metabolite production. The location of these two stains in the dendrogram was therefore questionable and they where subsequently removed from further analyses.

A binary matrix was made by visual examination of each peak and its associated UV–VIS spectrum in the HPLC chromatograms and the CIA dendrograms. On no occasion were there metabolites produced on DG18 or PDA + DN that were not found in DRYES, however, some metabolites were easier to detect on DG18 due to a qualitatively simpler metabolite profile. The matrix consisted of 137 recognizable metabolites for the 51 strains and was subjected to a Partial Least Squares Regression (PLS-R) (result not shown), which gave metabolites specific for the *A. malorum* strains, the *C. cetera* strain, the *A. infectoria* species–group strains and the *E. abundans* strains, respectively (see Table 3). All 51 chromatograms were analyzed for the production of known *Alternaria* metabolites and tested negative for AAL-toxins, alternariols, altersolanols, altenuenes, tentoxin and tenuazonic acid. On the other hand, HPLC-UV–VIS as well as HPLC-MS analyses showed that all 51 strains, including *E. abundans* (#30) and *A. malorum* var. *polymorpha* (#26), were able to produce infectopyrone and 4*Z*-infectopyrone. Altertoxin derived metabolites were restricted to the *A. infectoria* species–group, but not produced consistently throughout the species–group, while macrosporin was found in three *A. malorum* strains. Novae-zelandin production was shared by *E. abundans* and the *A. infectoria* species–group, but again not produced consistently throughout the species–group. *A. malorum* and *C. cetera* had a number of metabolites of unknown structure in common (e.g. RI value 694), but also produced unknown metabolites specific to each species (e.g. RI values 715 and 894, respectively). *E. abundans* did not produce any known metabolites, except for the infectopyrone, 4*Z*-infectopyrone, and novae-zelandin A, and only few species specific metabolites (e.g. RI values 691 and 779a) were detected.

Based on the combined results of the CIA and the PLS-R, a matrix that consisted of 49 strains [excluding *E. abundans* (#30) and *A. malorum* var. *polymorpha* (#26)] and 124 known and unknown metabolites (excluding infectopyrones and other consistently produced metabolites) was constructed. The matrix was subjected to cluster analysis and the resulting dendrogram is shown in Fig. 4. The dendrogram shows a division of the 49 strains into two clusters, A (in light grey) and B. Cluster A contains the type culture of *E. abundans* (#31), the type culture of *C. cetera* (#20) together with six *A. malorum* strains in sub-cluster A1 and two *A. malorum* strains (#22 and #28) in sub-cluster A2. Cluster B, holding all the 39 strains of the *A. infectoria* species–group, could be divided into two sub-clusters with type cultures of *A. photistica* (#05) and *A. triticina* (#02) as outliers. Sub-cluster B1 contained six *A. infectoria* species–group strains and the type cultures of *A. triticimaculans* (#08), *A. intercepta* (#12) and *A. viburni* (#12) (marked with ♦), while the majority of the *A. infectoria* species–group strains clustered closely together in sub-cluster B2. Both B sub-clusters could be further divided based on production of different metabolites. The color-coding of strains in Fig. 4 is referring to different haplotypes.

The result of a principal component analysis of the 39 *A. infectoria* species–group strains and 79 metabolites (given as their RI

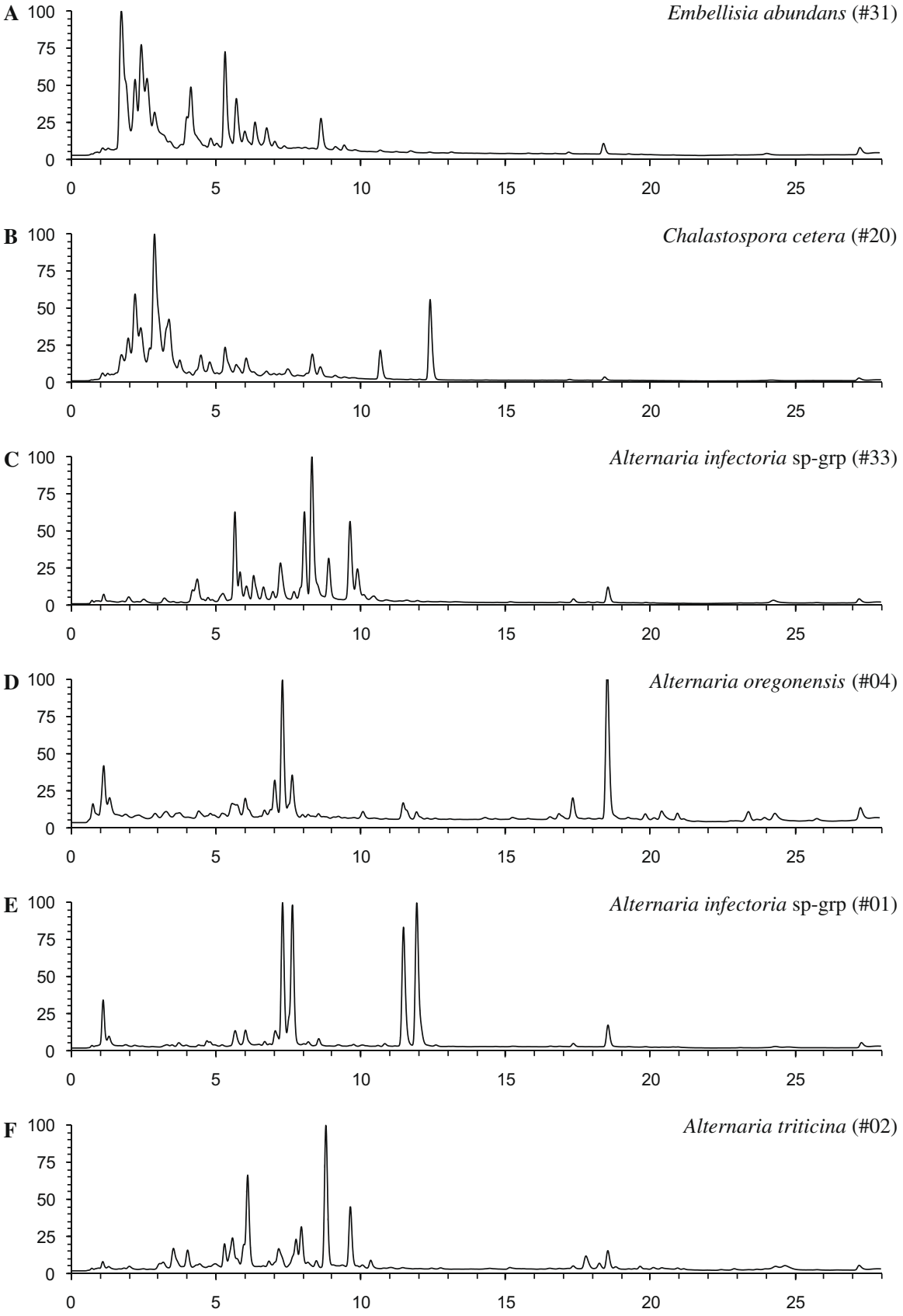

Fig. 3. HPLC chromatograms (wave length 210 nm). (A): *Embellisia abundans* (#31), (B): *Chalastospora cetera* (#20) and four strains belonging to the *Alternaria infectoria* species group; (C): *A. infectoria* species–group (#33), (D): *A. oregonensis* (#04), (E): *A. infectoria* species–group (#01) and (F): *A. triticina* (#02).

values) is shown in Fig. 5. It shows the association between metabolites and the strains that produce them. Metabolites common to most strains are located in the center, while metabolites specific to a few strains are located around the perimeter together with

Table 3
Production of known metabolites and selected, unknown metabolites, given by their retention index (RI) value, from *Alternaria infectoria* species–group, *A. malorum*, *Chalastospora cetera* and *Embellisia abundans*.

Metabolite (RI)[a]	*A. infectoria* sp–grp (39)	*A. malorum* (9)	*C. cetera* (1)	*E. abundans* (2)
Altertoxin derived (855)[b]	31	–	–	–
Altertoxin derived (820)	9	–	–	–
Altertoxin derived (846)	4	–	–	–
Infectopyrone (839)[c]	39	9	1	2
4Z-Infectopyrone (824a)	39	9	1	2
Infectopyrone derived (706)	30	2	–	–
Infectopyrone derived (713b)	23	1	–	–
Macrosporin (1068)	–[f]	3	–	–
Novae-zelandin B (892)	29	–	–	–
Novae-zelandin A (680a)	19	–	–	2
Novae-zelandin derived (726)	6	–	–	–
569	19	–	–	–
950[d]	13	–	–	–
691	–	–	–	1
779a	–	–	–	1
715	–	5	–	–
894	–	–	1	–
694	–	7	1	–
1010	–	4	1	–
642	–	7	1	1
1120	–	6	1	1
1076[e]	39	7	1	2
1047	35	5	1	1
752	26	5	–	1

[a] RI: retention index value, calculated by the HPLC from retention time.
[b] Same as metabolite 3 in Andersen and Thrane (1996).
[c] Same as metabolite 2 in Andersen and Thrane (1996).
[d] Same as metabolite 5 in Andersen and Thrane (1996).
[e] Same as metabolite 6 in Andersen and Thrane (1996).
[f] Not detected.

the strains producing them. From Fig. 5 it can be seen that strains in sub-cluster B1 in Fig. 4 (marked with ♦) produced a large number of metabolites including the altertoxins and novae-zelandins, whereas strains in sub-cluster B2 produce fewer metabolites and not altertoxins or novae-zelandins. On the other hand, strains in B2 produce metabolites of unknown structure (e.g. RI values 569, 706, 713b, 752, 813), which are not produced by strains in B1. In general, many individual metabolites of unknown structure were found to be specific to only one or a few strains in the *A. infectoria* species–group, which hampered a clear grouping.

3.4. Molecular cladification

The obtained sequences of *gpd* were 444–446 bp for *A. photistica* and the *A. infectoria* species–group and of 424 bp for *A. malorum*, *E. abundans,* and *C. cetera.* The aligned *gpd* sequences contained one intron of approximately 114 bp. The alignment dataset of all the strains contained 456 bp with 131 variable sites of which 85 were parsimony informative. Sequences of *tef-1α* were 437–440 bp containing two introns of approximately 250 bp in total. The *tef-1α* alignment dataset consisted of 443 bp containing 113 variable sites of which 65 were parsimony informative. The obtained ITS sequences were 490 bp for *A. photistica*, 519–525 bp for the *A. infectoria* species–group strains, 533–534 bp for the *A. malorum* strains and 523 bp and 525 for the *E. abundance* and *C. cetera* strains, respectively. The ITS sequence for *A. photistica* was smaller than those of the remaining strains due to a major deletion in ITS1. The ITS alignment dataset of all the strains contained 544 bp with 106 variable sites of which 60 were parsimony informative. The ITS and *tef-1α* dendrograms gave the same major division as the *gpd* dendrogram, but with lower resolution (data not shown). Strains that were identical in one gene sequence were nearly always different in another. Molecularly, all the *A. infectoria* species–group strains were mutually similar, but never identical, except for *A. infectoria* species–group strains (#10 and #11), which have identical sequences in all three tested genes.

Fig. 6 shows an unrooted dendrogram for all 51 strains of the concatenated ITS, *tef-1α* and *gpd* sequences using maximum likelihood in RaxML. It shows two major clades, one with 38 *A. infectoria* species–group strains and with *A. triticina* (#02), three strains of *A. infectoria* species–group (#09, #48 and #51) and *A. photistica* as outliers and another clade with all *A. malorum*, *C. cetera* and *E. abundans* strains (in light grey). Within the latter clade, *A. malorum* var. *polymorpha* (#26) could not be distinguished from the remaining eight *A. malorum* strains. Nearest neighbors of *A. malorum* were *C. cetera* and *E. abundans*. The degree of variability within the *A. infectoria* species–group proved to be limited in all genes.

Fig. 7 shows the nucleotide differences between 38 strains in the *A. infectoria* species–group [excluding *A. photistica* (#05)]. As seen in Fig. 7, most nucleotide differences in the three genes were observed in the spacers and introns, although there were some mutations in the coding region of *gpd*, which all occurred on the third codon position often with a silent C to T substitution. Table 4 shows alignment data for the three genes. Internal ITS alignment of the 38 strains in the *A. infectoria* species–group resulted in 40 variable sites of which 35 were parsimony informative and located in either the ITS1 or ITS2. The *tef-1α* alignment resulted in 113 variable sites of which 53 were parsimony informative. The *gpd* alignment showed 109 variable and 59 parsimony informative sites of which 18 were located in the 114 bp intron.

Using DnaSP on the *gpd* alignment data of the 38 strains in the *A. infectoria* species–group, haplotypic groups were defined and are given in Table 2. Most haplotypic groups contained only one strain except for haplotype 2 (#3, #17 and #43), haplotype 6 (#7, #32 and #47), haplotype 9 (#13, #19, #38 and #41), haplotype 15 (#35–37, #39, #46, #50 and #52) and haplotype 22 (#10–11), resulting in 24 distinct haplotype groups. Fig. 8 shows the haplotype network. The standardized Index of Association (I_A^S) of the

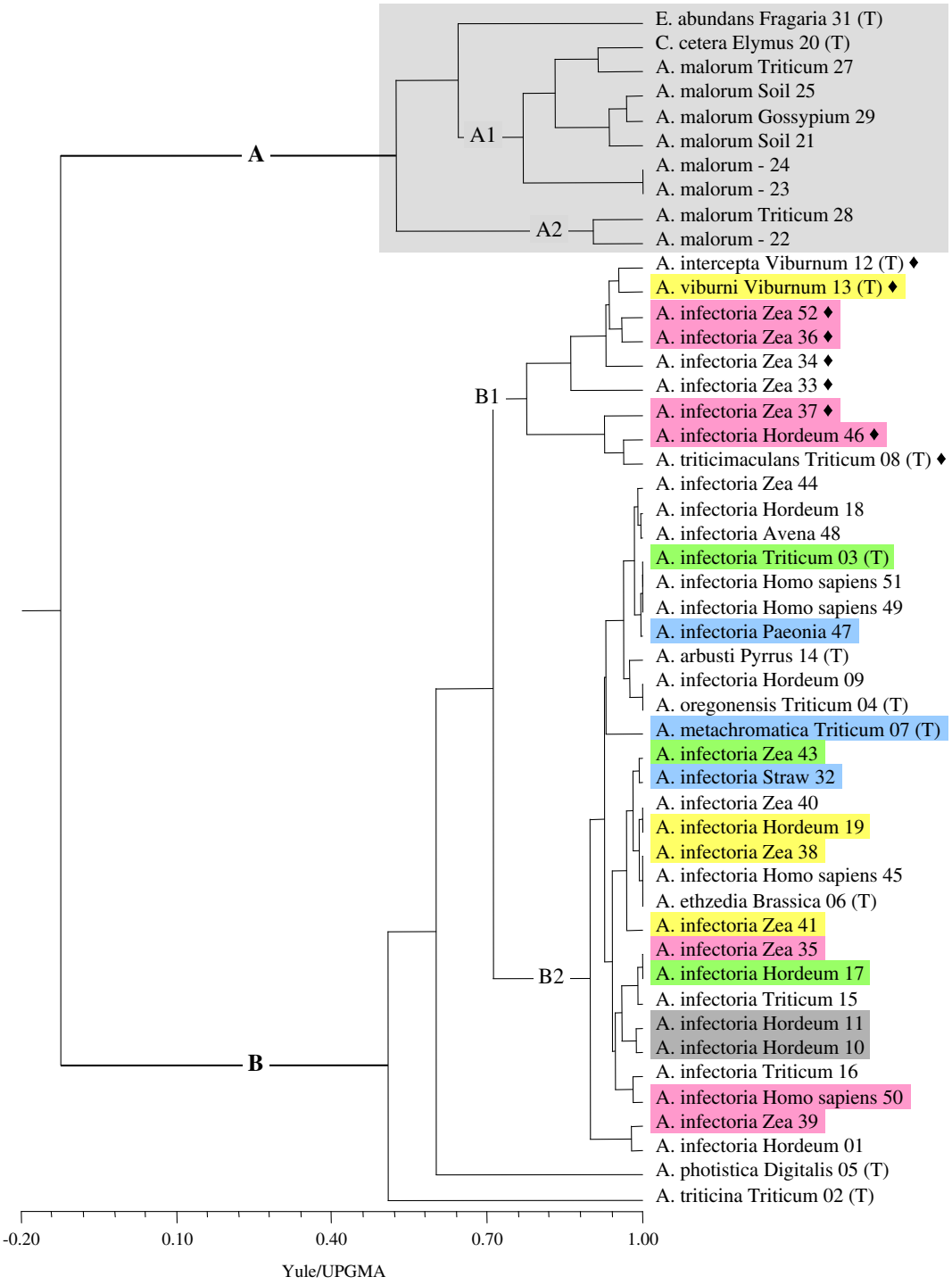

Fig. 4. Dendrogram based on a cluster analysis of 49 metabolite profiles (1 *Embellisia abundans*, 1 *Chalastospora cetera*, 8 *Alternaria malorum* and 39 strains belonging to the A. *infectoria* species–group). Color-coding in the B cluster corresponds to haplotype groups given in Table 2 and Fig. 8. Strain labels: strain ID/host/strain number/type culture. Dendrogram calculated using the Yule correlation coefficient and UPGMA as the clustering method. Axis shows the correlation coefficient from −1 to 1.

same *A. infectoria* species–group strains showed a tendency towards recombination events, $(I_A^S) = 0.1627$. LIAN v3.5 was used to calculate the standardized Index of Association with 1,000,000 Monte Carlo samplings. The neighbornet splitstree of *gpd* alignment data (not shown) of the *A. infectoria* species–group showed mostly a treelike structure. The network also showed conflicting phylogenetic trees (histories) that can not be shown with a bifurcating tree. Conflicting phylogenetic signals can occur by recombination or by convergent substitutions and can not be distinguished by looking at the network alone (Salemi et al., 2008). However, a *phi*-test was able to detect the presence of recombination in aligned sequences. Repeated *phi*-test calculations after removing single sequences from the alignment showed the presence of recombinants. When the *p*-value increased till 0.05 or more, it was obvious that the recombinants were deleted from the alignment. Table 4 shows the *p*-values for the three genes in the *phi*-test of the 38 *A. infectoria* species–group strains [excluding *A. photistica*]. ITS and *tef-1α* had *p*-values > 0.05, which indicated

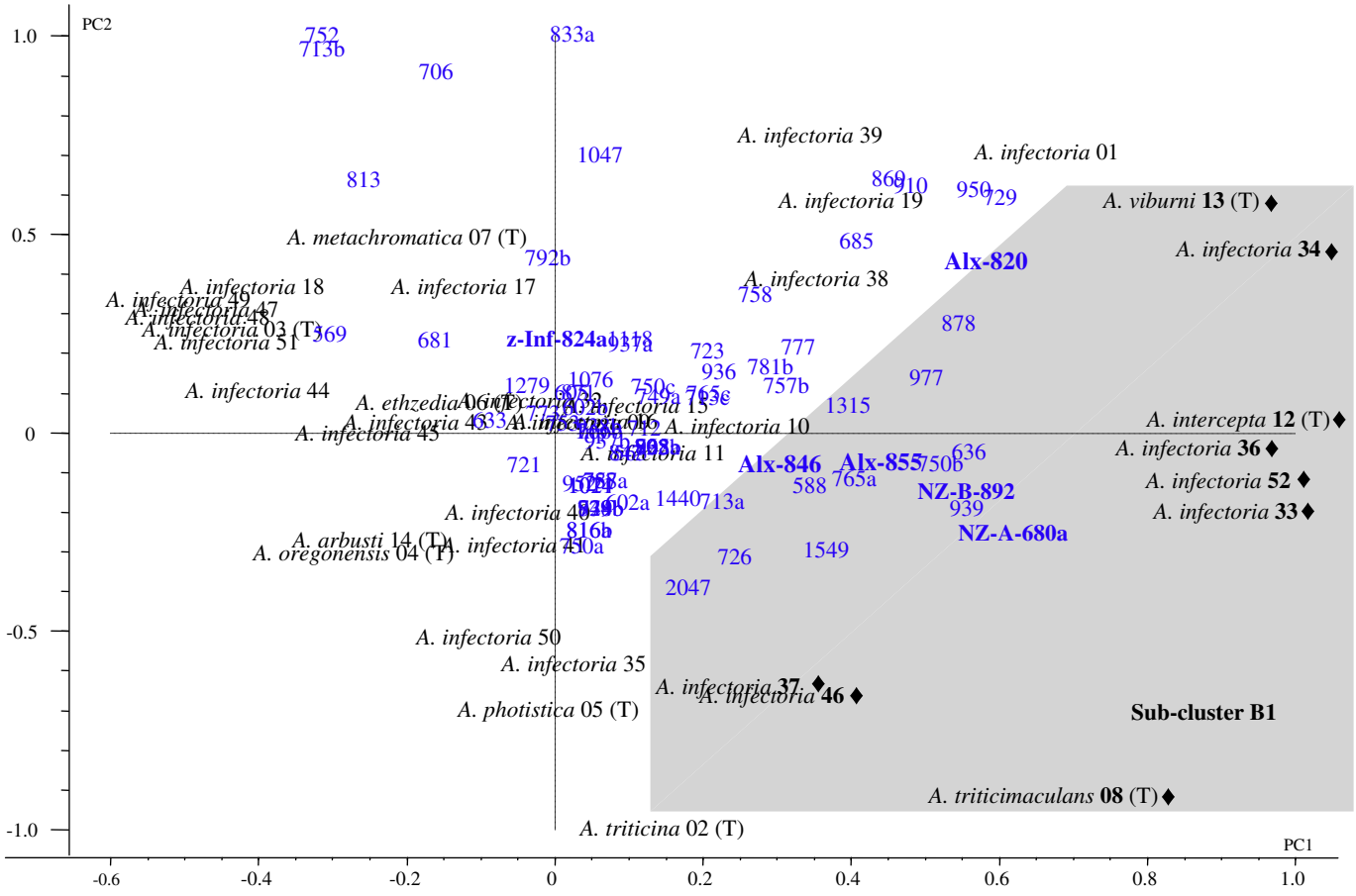

Fig. 5. Loadings plot based on a principal component analysis of 79 individual metabolites (39 strains belonging to the *A. infectoria* species–group). Strains are in black and metabolites in blue. Metabolites of known structure: Alx: altertoxin derivatives; NZ: novae-zelandin derivatives; z-Inf: infectopyrone derivate. Metabolites of unknown structure are only given by their Retention Index (RI) values calculated from their retention time on HPLC. Strain labels: strain ID/strain number/type culture. Strains in the grey box marked with ♦ correspond to sub-cluster B1 in Fig. 4. Axes are score values.

the absence of recombination. However, the *p*-value of *gpd* was 7.5×10^{-4} suggesting the presence of recombination events in this gene. After deleting strains #08, #45 and #51 (CBS 578.94, CBS 102692 and CBS 110804, respectively), the *phi*-test showed no significant evidence of recombination in the *gpd* data ($p > 0.05$) and therefore these three strains were considered to be recombinants.

4. Discussion

At genus level, the *Alternaria infectoria* species–group could clearly be separated from the *A. malorum/C. cetera/E. abundans* group based on morphology as well as chemical classification and molecular cladification. The results show that strains morphologically identifiable as *A. infectoria* species–group produced altertoxins and novae-zelandins and yielded ITS, *gdp* and *tef-1α* sequences that were different from those of the *A. malorum/C. cetera/E. abundans* group (Figs. 1, 4 and 6 and Table 3). *E. abundans*, on the other hand, could only be segregated from the *A. malorum/C. cetera* group by morphology (Fig. 1), but not with any confidence by molecular or chemical means. Besides, the chemical similarity turned out to be too large and the number of species/strains used proved to be too few to speculate on the placement of *Embellisia*, compared to *A. malorum/C. cetera* group. Other studies based on ITS, SSU and *gpd* data, show species of *Embellisia* are scattered among genus *Alternaria* as well as genus *Ulocladium* despite its distinct morphology (Pryor and Bigelow, 2003). In contrast, *C. cetera* could not be segregated from the *A. malorum*/*A. malorum* var. *polymorpha* group by morphological, molecular, or chemical means (Figs. 1, 4 and 6 and Table 3). Two *A. malorum* strains (#23 and #24) were distinct and identical in all three methods. The other *A. malorum* strains yielded metabolite profiles that were similar, but not identical to each other and to that of *C. cetera*. Morphologically, *A. malorum* and *C. cetera* showed the same general sporulation pattern, but with some variation in conidial size and septation. The polyphasic data in this study shows that *A. malorum* var. *polymorpha* and the eight *A. malorum* strains, do not belong in the *A. infectoria* species–group as proposed by Braun et al. (2003), but suggest they belong to the same genus as *Chalastospora cetera*, however, as several distinct species.

The production of infectopyrones and a pair of compounds with unknown structure (RI values 1047 and 1076) by all genera in this study corroborates the close relationship found in the molecular analyses, but on the other hand, some species of *Ulocladium*, which are phylogenetically more related to small-spored *Alternaria* (Pryor and Bigelow, 2003), also produce infectopyrones (Andersen and Hollensted, 2008) and these metabolites may be more widespread in *Pleosporaceae*. Furthermore, this is the first report on the production of infectopyrones by *A. malorum*, *C. cetera*, and *E. abundans* and the production of macrosporin and novae-zelandin A by *A. malorum* and *E. abundans*, respectively.

At species level, the 10 *Alternaria* type cultures representing morphological species within the *A. infectoria* species–group were

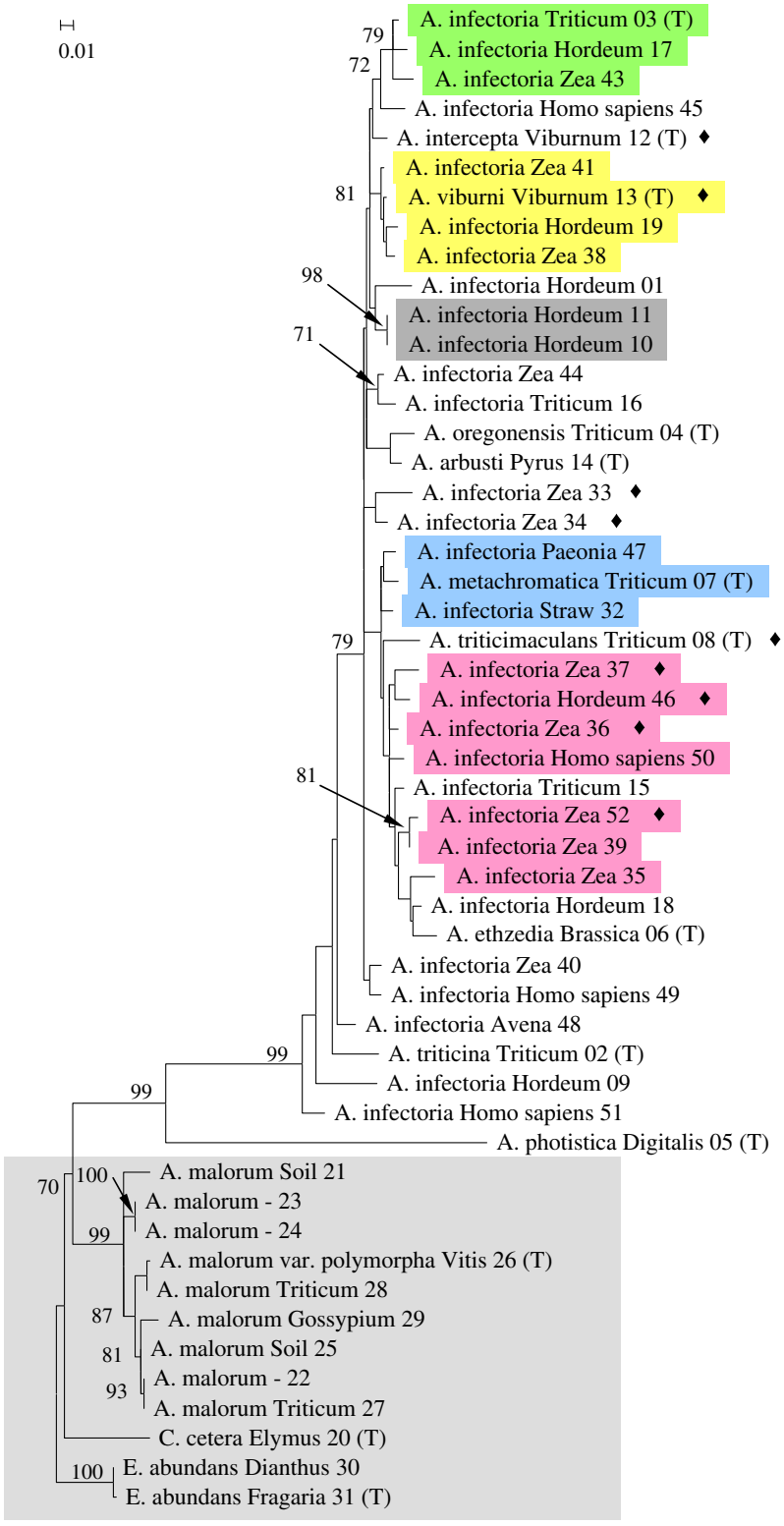

Fig. 6. Unrooted consensus dendrogram based on 51 strains (2 *Embellisia abundans*, 1 *Chalastospora cetera*, 39 *Alternaria infectoria* species–group, 8 *A. malorum* and 1 *A. malorum* var. *polymorpha*). Maximum likelihood tree of 3 partial genes (ITS, *gpd* and *tef-1α*) constructed using RaxML (Cipres webserver). Bootstrap values > 70% are indicated. Color-coding in the *A. infectoria* species-group clade corresponds to haplotype groups given in Table 2 and Fig. 8. Strains marked with ♦ correspond to cluster B1 in the chemical analysis. Strain labels: strain ID/strain number/type culture.

located in different sub-clades depending on the molecular sequence examined, but with *A. photistica* (#05) and *A. triticina* (#02) as outliers. With each individual gene, variability was largely random, judging from low bootstrap values and from obtaining different groupings when different algorithms were used for tree reconstruction. When genes were concatenated, *A. viburni* (#13) clustered at 81% bootstrap support with three strains (#19, #38 and #41) identified as *A. infectoria* species–group *sensu* Simmons.

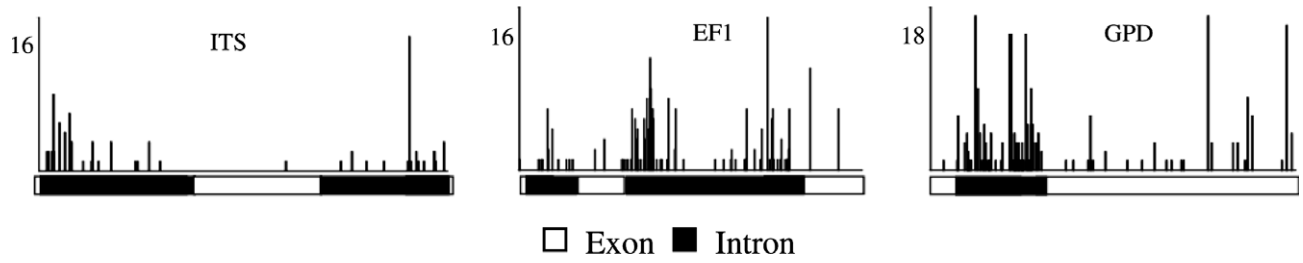

Fig. 7. Mutations at each position in the aligned ITS, *tef-1α* and *gpd* sequences of the 38 *A. infectoria* strains (except *A. photistica* #05) with the predicted exons and introns.

The single type cultures were not unambiguously separated from the core of *A. infectoria*. The same pattern was seen in the chemical data with *A. photistica* (#05) and *A. triticina* (#02) as outliers. No distinct groups or clades were formed in the molecular analyses, while there was a certain grouping in metabolite profiles and metabolite families (Figs. 3–5 and Table 3). Some *A. infectoria* species–group strains were able to produce altertoxin derivatives, while others produced metabolites of unknown structures, but not altertoxins. No distinct morphological groups were seen either among the strains in the *A. infectoria* species–group. Morphology showed that basically each strain was a taxon in its own right. Lastly, no groupings or correlations could be found between proascoma formation, ability to grow at 37 °C, host or geographic origin and haplotypes, metabolite production or morphological identity.

Our original hypothesis was that taxa in *Lewia*/*A. infectoria* species–group were sexual fungi and that molecular sequence analyses and metabolite profiling would yield a number of groups according to the genealogical concordance phylogenetic species recognition (GCPSR) (Taylor et al., 2000). Our data, however, indicate that only three strains in the *A. infectoria* species–group show evidence of recombination and that several isolates are able to produce proascomata in axenic culture. Since several taxa in the *A. infectoria* species–group have been shown to produce ascomata with viable ascospores in axenic cultures (Kwasna and Kosiak, 2003; Simmons, 2007; unpublished results), *Lewia*/*A. infectoria* species–group must, at least in part, be homothallic and the purpose of ascoma formation in nature could be a survival strategy. The high similarity in nucleotide sequence amongst the *A. infectoria* species–group strains (Fig. 6), suggests that most strains are clonal and may have derived via mutations from one common ancestor similar to the arbuscular mycorrhizal fungi (Rosendahl, 2008).

Several studies (reviewed in Taylor et al., 2000; O'Donnell et al., 2004) show an increase in numbers of taxa, when going from morphological species recognition via biological recognition to GCPSR, which corresponded with either geographic origin or hosts. In our study we see the opposite: molecular cladification yields the lowest number of taxa in the *A. infectoria* species–group (*A. photistica* and one phylogenetic taxon), while the chemical classification gives more (*A. photistica* and *A. triticina* and two chemically different taxa) and with morphological appearance giving the highest number (38 morphologically different taxa). Applying GCPSR to the *A. infectoria* species–group would lead to synonymizing of all morphological species in the *A. infectoria* species–group under one name: *A. infectoria* Simmons. Alternatively, morphological species recognition could be applied and strains in the *A. infectoria* species–group would represent new "emerging" species that require a name and a formal description. But according to Taylor et al. (2000) and Rosendahl (2008), GCPSR can only be applied to sexual/heterothallic fungi, not to homothallic/clonal strains, so neither of the two approaches (one species or 38 species) is workable.

In practice, however, there is a regular need for identification of *Alternaria* isolates, because they have acquired different abilities in nature, which affect us negatively. Some isolates have been encountered as opportunistic human pathogens, others as plant pathogens and others again are saprotrophic on cereals producing biologically active metabolites. Artificial identification systems based on any stable differentiation characters (e.g. PCR, AFLP, metabolite profiles, sporulation patterns obtained under standardized conditions) still play an important role in taxonomy. Strains in the *A. infectoria* species–group show characteristic phenotypical traits, which can be detected, recognized, and used for identification. Depending on the users needs, identification of taxa in the *A. infectoria* species–group can be done to different levels. In medical mycology, molecular identification using ITS is fast, well-known and often the only method to obtain the correct diagnosis for isolates in the *A. infectoria* species–group, since strains from human lesions rapidly loose their ability to sporulate *in vitro*. Strains used in this study that originated from human skin lesions sporulated poorly, even under optimal conditions, and went sterile after one or two transfers. However, they still maintained their ability to produce all the characteristic metabolites in spite of their vegetative or yeast-like growth. Concerning alternarioses in humans or animals, generally only identification to species–group level is needed, since the same medical treatment (e.g. itraconazole) is likely to be applicable regardless of taxon identification within the *A. infectoria* species–group (Brasch et al., 2008; Dye et al., 2009). In plant pathology, phytosanitary, and quarantine, on the other hand, ITS sequencing is not enough to identify a known pathogen or discover a new pest that requires quarantine. With our current knowledge, described plant pathogens like *A. triticina*, *A. viburni,* and *A. intercepta* can be distinguished from other taxa of the *A. infectoria* species–group using morphology. In the cladistic analyses, *A. triticina* (#02) grouped with different taxa in the *A.*

Table 4
Alignment data set for 38 strains in the *Alternaria infectoria* species–group, except *A. photistica,* of the three genes with number of mutations, parsimony informative mutations, sites and *p*-value in *phi*-test.

	Total number of sites (gaps/missing)	Total number of mutations	Number of parsimony informative sites	Parsimony informative mutations (%)[a]	Parsimony informative sites (%)[b]	*phi*-Test *p*-value[c]
gpd	456 (40)	109	59	54.1	12.9	$7.5 \cdot 10^{-4}$
tef-1α	443 (17)	113	53	46.9	12.0	0.15
ITS	546 (71)	40	35	87.5	6.4	0.41

[a] Percentage is calculated using the total number of mutations.
[b] Percentage is calculated using the total number of sites.
[c] *p*-Value < 0.05 shows presence of recombination.

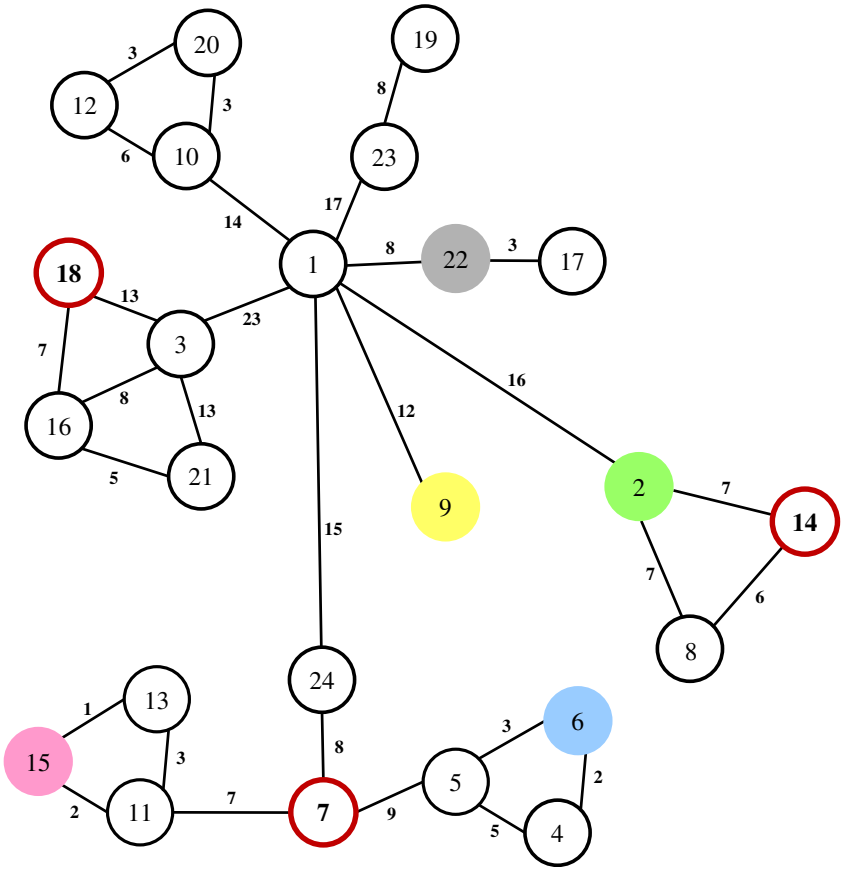

Fig. 8. Haplotype network of *A. infectoria* species–group strains (except *A. photistica* #05) based on maximum parsimony tree of *gpd* sequences. The circles represent the 24 different haplotype groups, which are given in Table 2. The lines between the groups connecting the haplotypes show the number of nucleotides differing. Circles with solid colors (haplotypes 2, 6, 9, 15 and 22) contain more than one strain and circles with red lines (haplotypes 7, 14 and 18) show the position of the recombinant strains.

infectoria species–group depending on the chosen DNA sequence, but was an outlier chemically, having a different metabolite profile. Further research may yield *A. triticina* specific metabolites that can be used to facilitate identification. In food safety, taxa in the *A. infectoria* species–group regularly contaminate cereal grain (Andersen et al., 1996; Pitt and Hocking, 1997; Kosiak et al., 2004; Perelló et al., 2008). The most urgent need is to know what secondary metabolites and other biologically active compounds are produced in the cereals like wheat, barley, and maize. Since current knowledge does not allow connections between metabolite profiles and morpho-species to be made, chemical analyses are needed.

The results presented in this study show that these household genes (ITS, *tef-1α* and *gpd*) do not reflect ecology, secondary metabolism or morphology of the *A. infectoria* species–group and that molecular cladification and phylogeny cannot predict pathogenicity, host specificity or mycotoxin production. Concerning the classification and the systematic placement of the strains and morpho-species in the *A. infectoria* species–group, a polyphasic approach is needed, but there are inconsistencies between the different taxonomic features and we therefore refrain from recommending any taxonomic changes at this point in time.

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Polyphasic characterization of *Phoma pomorum* isolated from Danish maize

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Abstract

Strains of the genus *Phoma* are often isolated from various environmental samples including cereals and maize. In the present study we performed a polyphasic characterization of strains isolated from Danish samples derived from whole plant material collected at harvest. All strains were isolated using a newly developed isolated medium and identified morphologically as *P. pomorum*. This species is placed in the *Phoma* section *Peyronellaea* and strains of other members of this section were also included in the present study. Sequence analysis of the internal transcribed spacer region (ITS) was able to group the different species with the Danish *P. pomorum* grouping together with *P. pomorum* reference strains isolated from other sources.

The metabolite production on dichloran Rose Bengal yeast extract sucrose agar (DRYES) was analyzed and the strains were clustered using an in-house chemical image analysis (CIA) program. The resulting tree showed three clusters, one containing all *P. pomorum* strains, one containing all *Epicoccum nigrum* strains and finally a large cluster containing strains of the remaining species, which could not be differentiated due to insufficient metabolite production. The separation of *P. pomorum* from the other species resulted mainly from the ability of the species to produce isocoumarins. Several isocoumarins were produced by *P. pomorum* strains with diaportinic acid as the predominant analogue, but diaporthin, dichlorodiaporthin, diaportinol, citreo-isocoumarin, 6-methyl citreo-isocoumarin and citreo-isocoumarinol were also identified. This is the first time that the *Phoma* genus has been reported as a producer of isocoumarins.

Keywords: Phylogeny, chemotaxonomy, secondary metabolites, extrolites, mycotoxins

Introduction

The asexual genus *Phoma* contains several cosmopolitan species present in a wide range of commodities. The genus has recently been revised using culturing techniques and morphological data in a series of papers by Gerhard Boerema and co-workers resulting in the "*Phoma* identification manual", which describes more than 220 inter- and intraspecific taxa divided in nine sections (Boerema et al., 2004). The actual number of taxa may, however, be much higher as only a portion of the thousands of species described in literature have been verified in vitro (Aveskamp et al., 2008).

At the Department of Systems Biology, Technical University of Denmark, we have during the last four years examined the mycobiota and some of their mycotoxins in Danish maize plants (Storm et al. 2008) and in this period we frequently observed *Phoma* spp. in the examined samples. *Phoma* spp. have also been observed in other studies of the mycobiota of maize, but the isolated strains have usually not been identified to species levels, but instead listed as *Phoma* spp. (Ono et al., 2002; Schumann et al., 1991). Several *Phoma* species, including *P. sorghina*, *P. zeae-maydis* and *P. subglomerata* have been associated with a disease in maize with similar symptoms as *Phaeosphaeria* leaf spot in Brazil (do Amaral et al., 2004; do Amaral et al., 2005), but to our knowledge these species have not been reported from European maize yet. The occurrence of *Phoma* spp. in food and feed products is of concern to consumers and growers due to possible production of bioactive secondary metabolites, although there are limited reports on metabolite production by identified *Phoma* species. *P. sorghina* has been reported as a producer of tenuazonic acid (Shephard et al., 1991), a potent bioactive compound normally produced by several small spored *Alternaria* species (Andersen et al., 2002). *P. lingam* (sexual stage *Leptosphaeria*

60 *maculans*), the causative agent of blackleg disease of brassica oilseeds, has also been reported as a
61 producer of several bioactive compounds (Pedras and Chumala, 2005).

62 To isolate and later identify *Phoma* strains from maize we used a recently developed a semi-
63 selective isolation medium, potato carrot agar with manganese (PCA-Mn) (Sørensen et al., 2009).
64 All the isolated strains were identified morphologically as *P. pomorum*, which is accommodated in
65 *Phoma* section *Peyronellaea* (Boerema, 1993). There is little available information on *P. pomorum*
66 physiology, metabolite production and phylogeny and we therefore decided to perform a polyphasic
67 characterization of the strains isolated from Danish maize. Strains of some of morphologically
68 similar species also accommodated in the *Peyronellaea* section, viz. *P. americana, P. eupyrena, P.*
69 *gardeniae, P. glomerata, P. multistrata, P. sorghina* and *P. zeae-maydis* (Aveskamp et al., 2009)
70 were also included in the study in addition to *Epicoccum nigrum* (Syn. *P. epicoccina*).

71 One of the tools we wanted to use in the characterization of the *P. pomorum* strains was
72 chemotaxonomy based on production of secondary metabolites. Chemotaxonomy has previously
73 been used to differentiate terrestrial and marine *Phoma* spp. isolates using HPLC with UV and MS
74 detection (Osterhage et al., 2000). Likewise metabolite profiling has been used to group strains of
75 the blackleg fungus *P. lingam* (sexual stage *Leptosphaeria maculans*) obtained diverse parts of the
76 world (Pedras and Biesenthal, 2000). Through metabolite profiling of *P. pomorum* strains from
77 maize we would also be able to assess whether this species is able to produce potentially harmful
78 bioactive compounds during maize infections.

79

80 **Materials and methods**

81 *Fungal strains*

A total of 22 *P. pomorum* strains isolated from maize samples derived from whole maize plants collected at harvest were included in this study. The samples were collected from farms distributed across the 29.777 km² peninsula Jutland, Denmark. Further, 43 strains were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and PD (Dutch Plant Protection Service, Wageningen The Netherlands) for reference, including *P. americana* (7), *P. eupyrena* (2), *P. gardeniae* (2), *P. glomerata* (8), *P. multistrata* (6), *P. pomorum* (5), *P. pomorum* var. *cyanea* (1), *P. sorghina* (4), P. *zantedeschiae* (1), *P. zeae-maydis* (1) and *E. nigrum* (6). Five *P. pomorum* strains isolated from various sources outside Denmark were also included together with one *P. pomorum* var. *cyanea* strain (**Table 1**). Strains are preserved in the CBS collection, The Netherlands, or IBT collection, Department of Systems Biology, DTU, Denmark.

DNA extraction, PCR amplification and sequencing

Strains were grown on oat meal agar (OA) (Samson et al., 2004) for one week in the dark and DNA was isolated using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to manufactures protocol and stored at -20 °C. Amplification of the internal transcribed spacer region (ITS) was performed using the V9G forward primer (de Hoog and van den Ende, 1998) and the ITS4 reverse primer (White et al., 1990). PCR and sequencing methodologies followed the procedures described previously (Woudenberg et al., 2009). A consensus sequence was computed from the forward and reverse sequences with the BioNumerics v4.61 software package (Applied Maths, St-Martens-Lathem, Belgium). The obtained sequences have been submitted to GenBank (**Table 1**).

104 *DNA sequence alignment and analysis*

105 The obtained sequences were aligned using BioNumerics and adjusted manually where necessary.
106 A Bayesian tree inference (BI) analysis was performed with MrBayes v3.1.2 (Huelsenbeck and
107 Ronquist, 2001). Using the MrModeltest software (Nylander, 2004), the optimal substitution model
108 for this dataset was found to be symmetrical model with inverse gamma rates and equal dirichlet
109 base frequencies. One tree was saved per 100 generations and the temperature value was set at 0.2.
110 The number of generations was set at 2.5M, but the run was automatically ended when the standard
111 deviation of split frequencies was below 0.01. To avoid suboptimal trees being taking into account
112 for the consensus tree, a burn-in of 25% of the saved trees was used.

113 To obtain further measure of branch support was obtained by conducting a maximum likelihood
114 (ML) analysis using RAxML (randomized axelerated maximum likelihood) software (Stamatakis et
115 al., 2008) through the CIPRES Website (www.phylo.org). The symmetrical model was omitted
116 here, as RAxML implements only the GTR substitution model. The robustness of the tree was
117 evaluated by bootstrap. The number of replicates that is sufficient provide stable bootstrap values is
118 automatically determined by the software (Stamatakis et al., 2008).The resulting ‘‘50% majority
119 rule consensus’’ trees were printed with TreeView v1.6.6 (Page, 1996).

120

121 *Metabolite extraction*

122 For metabolite analysis, each strain was transferred aseptically with three point inoculated onto the
123 growth medium and incubated for two weeks at 25°C in the dark. To find the optimum medium for
124 metabolite production by *P. pomorum* (strains IBT 41376, 41453 and 41454) the following media
125 were tested: dichloran Rose Bengal yeast extract sucrose agar (DRYES), dichloran 18% glycerol

agar (DG18), malt extract agar (MEA), oatmeal agar (OA), yeast extract agar (YES), potato dextrose agar (PDA), czapek yeast extract agar (CYA), czapek yeast extract agar 20% sucrose (CYA-S), rice corn steep agar (RC) (Samson et al., 2004) and Wickerhams antibiotic test medium (WATM) (Raper and Thom 1949). The metabolites were extracted using a modified version of the micro-scale extraction method (Smedsgaard, 1997). In brief: three 6-mm agar plugs were cut from the centre of each of the three colonies and placed in a 2-mL vial. Plugs were first extracted with 1.0 mL ethyl acetate containing 0.5% formic acid (v/v) and successively with 1 mL isopropanol, both ultrasonically for 60 min. The extracts were transferred to clean 2-mL vials, evaporated to dryness *in vacuo*, re-dissolved ultrasonically with 400 µL methanol, and filtered through 0.45-µm PFTE filters (National Scientific Company, Rockwood, TN, USA) into clean 2-mL vials prior to HPLC analysis.

HPLC-UV-VIS analyses

The extracted metabolite were analyzed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped a diode array detector collecting 2 ultraviolet-visible (UV-VIS) spectra per second from 200 to 600 nm. 5 µL extract was inject and separated on a 2×100 mm Luna 3 µm C18(II) (Phenomenex, Torrance, CA, USA) at 40ºC using a linear water-acetonitrile gradient and a flow of 0.4 ml/min. The gradient started at 15% acetonitrile, reached 100% in 20 min and was held for 5 min. Both eluents contained 50 ppm trifluoroacetic acid. A homologous series of alkylphenones was analyzed as external retention time references and used to calculate a bracketed retention index (RI) for each detected peak (Frisvad and Thrane, 1987).

148 *Data treatment of metabolite profiles*

149 Metabolite profile data from HPLC-UV-VIS analyses were first treated with the Chemical Image
150 Analyses (CIA) program, which has used previously in analysis of *Alternaria* and *Ulocladium*
151 (Andersen et al., 2005; Andersen and Hollensted, 2008). In brief: The raw HPLC data files, which
152 are quantitative 2-D matrices (x-axis: time, Y-axis: wave length, value in matrix: UV-VIS
153 absorbance), were transferred from the HPLC to a standard PC and analyzed by CIA. No
154 manipulations or peak selections were made before processing. Each HPLC file was processed first
155 by a $\log_{10}$ scaling (to account for concentration differences among extracts), then a baseline
156 correction and finally an alignment (to account for drift in baseline and retention time among
157 identical metabolites in different runs). Each HPLC file was then compared to the other 64 HPLC
158 data files, pair-wise, giving a similarity value for each pair, which was entered into a new matrix.
159 The resulting 65×65 similarity matrix was then used to calculate a dendrogram using WARD
160 clustering method.

161

162 *Identification of isocoumarins*

163 Reference standards of diaportinic acid, diaporthin, diaportinol, dichlorodiaporthin, citreo-
164 isocoumarin and 6-methyl citreo-isocoumarin were available from previous studies (Larsen and
165 Breinholt, 1999; Nielsen and Smedsgaard, 2003). The metabolite standards were run on the HPLC
166 system with the same settings as for the fungal extracts. Retention time and extracted UV-VIS
167 spectra were used to identify the compounds in the fungal extracts. The identity of the compounds
168 were confirmed by high resolution MS detection, which was done on a Time Of Flight mass
169 spectrometer (Water-Micromass, Manchester, UK) scanning m/z 60-900 and m/z 100-2000 in two
170 separate scan functions at different skimmer settings (Nielsen et al., 2005). The system operated in

171 the positive mode and the compounds were detected using the $[M+H]^+$ or $[M+NH_4]^+$ ions with an
172 interval of mass ± 0.02 amu.

173

174 **Results and discussion**

175 *Molecular cladification*

176 The ITS alignment consisted of 29 sequences generated in this study and 32 obtained from
177 GenBank. This ITS alignment consisted of 495 characters including alignment gaps, of which 57
178 were variable. Sequences of *Phoma sorghina* (#60-63) were used as outgroup. The BI analysis run
179 of the ITS sequence matrix resulted in 570 trees, from which the burn-in was discarded and the
180 consensus tree and posterior probabilities were calculated. The topology and support values of the
181 BI tree were in congruence with those of the optimal tree obtained in the ML analysis.

182 The reconstructed phylogeny using the ITS dataset revealed ten clusters shown in **Figure 1**, which
183 largely corresponded with the taxa recognized by morphological studies (Aveskamp et al., 2009;
184 Boerema et al., 2004). *Phoma pomorum* is clustered in two separate groups, which differ only by
185 the presence of a single adenine-thymine substitution in the ITS2 region at position 448 (e.g.
186 GenBank FJ427057 vs. FJ427058). In both *P. pomorum* groups, isolates from Danish maize are
187 embedded. This grouping could however not be linked with their geographical origin, maybe
188 because the peninsula Jutland, from where the samples were collected, is relatively small (29.777
189 km²). Morphological observations of those strains did not reveal any characters that are congruent
190 with the presence of this SNP.

191 *Phoma pomorum* var. *cyanea* (#28), formerly known as *P. cyanea* (Jooste and Papendorf, 1981), is
192 a species that thus far has been reported only from South Africa. It is characterized by the

193 production of a bluish pigment in the hyphae, pycnidia and chlamydospores. Except for this feature,
194 the remaining morphological characters fit within the scope of *P. pomorum*. Also the ITS sequence
195 is identical to that of CBS 539.66 (#25), the strain that has been appointed as reference strain for *P.*
196 *pomorum* (Boerema, 1993). It is concluded therefore that *P. cyanea* is correctly reduced to a variety
197 of the older *P. pomorum*, as *P. pomorum* var. *cyanea*.

198

199 *Chemical classification*

200 DRYES was chosen as growth medium for chemical classification, because this medium was
201 qualitatively and quantitatively superior to DG18, MEA, OA, YES, PDA, CYA, CYA-S, RC and
202 WATM as the medium supported production of more metabolites, which were also produced in
203 higher amounts by the three representative *P. pomorum* strains tested (unpublished data). An
204 automated and unbiased chemical image analysis (CIA) of the HPLC-UV files was made, which
205 resulted in a dendrogram consisting of three major clusters (**Figure 2**). The top cluster consisted
206 solely of all *P. pomorum* strains, the bottom cluster consisted of all *E. nigrum* strains and one *P.*
207 *sorghina* strain (#63), while the middle cluster consisted of the remaining strains. The results
208 therefore show that the metabolite profile of *P. pomorum* is quite different from the other species.
209 *P. pomorum* var. *cyanea* did however not cluster together with the other *P. pomorum* strains, which
210 either indicate that it is not a *P. pomorum* or that the mutations causing its blue color also influence
211 its global metabolite production. Several small groups occurred in the *P. pomorum* cluster, which
212 was not linked to the two ITS sequence types or geographical origin.

213 *E. nigrum* had also a species specific metabolite profile, although one *P. sorghina* strain clustered
214 together with this species. The six *E. nigrum* strains and *P. sorghina* (#63) produced all high
215 quantities of a wide range of metabolites, which may have resulted in an injection overload, which

could not be handled by the CIA software. Visual inspection of the HPLC chromatograms showed that *P. sorghina* (#63) had only a few metabolites in common with the six *E. nigrum* strains and that this clustering may have resulted from the injection overload. Strains in the middle cluster produced only few metabolites compared to *P. pomorum* and *E. nigrum* (**Figure 3**), which troubled successful clustering of all species. All *P. multistrata* were however located in the same subcluster, as were the two *P. gardeniae* strains indicating that these two species have species specific metabolite profiles. Strains representing the remaining species were however more or less scattered in this middle cluster, indicating that the methodology used for chemotaxonomy in the present study could not be used to differentiate these species. This was however not the purpose of the study and we have therefore not optimized the growth medium, extraction solvents and HPLC settings for these species. These issues need therefore to be addressed in future studies aiming to explore the chemotaxonomical relationship of these species.

Identification of Phoma pomorum metabolites

Visual inspection of the HPLC-UV-VIS chromatograms showed that the *P. pomorum* strains produced a multiple compounds with identical spectra characteristic for the group of isocoumarins (**Figure 3**). Available isocoumarin reference standards from our metabolite collection at Department of Systems Biology were run and their retention index (RI) were used to tentatively identify them in fungal extracts. Their identity was then confirmed with high resolution MS by running reference metabolite standards as well as selected representative *P. pomorum* strains using the ions listed in **Table 2**. In this way citreo-isocoumarin, diaportinic acid, diaporthin and dichlorodiaporthin were identified in the *P. pomorum* extracts (structures shown in **Figure 4**). Trace amounts of diaportinol and 6-methyl citreo-isocoumarin (structures shown in **Figure 4**) could also

239 be detected by LC-MS, but not with HPLC-UV because these two compounds co-eluted with other
240 interfering compounds. A compound, which eluted in front of citreo-isocoumarin in both HPLC-
241 UV-VIS and LC-MS, was tentatively identified as citreo-isocoumarinol based on $[M+H]^+$ and
242 $[M+NH_4]^+$ ions, but as a reference standard of this metabolite was not in stock, the identity could
243 not be confirmed.

244 Citreo-isocoumarinol, citreo-isocoumarin, diaportinic acid and dichlorodiaporthin were produced by
245 all *P. pomorum* isolates in detectable amounts, but also by two *P. glomerata* isolates (#51 and #53).
246 Another *P. glomerata* (#49) strain produced also dichlorodiaporthin, whereas diaportinic acid was
247 also produced by one additional *P. glomerata* strain (#52). 22 *P. pomorum* strains produced
248 detectable quantities of diaporthin, which was not detected in other species. An unidentified
249 compound (RI 757) with the typical isocoumarin spectra, which was only produced by 11 *P.*
250 *pomorum* strains, was produced by several of the other species. Especially *P. americana* was a good
251 producer of this compound as it was detected in six of the seven strains with (#37) being the sole *P.*
252 *americana* strain not producing this compound.

253 Diaportinic acid, diaportinol, diaporthin and dichlorodiaporthin have previously only been reported
254 from *Penicillium nalgiovense* (Larsen and Breinholt, 1999), whereas citreoisocoumarins have been
255 isolated from several species of *Penicillium* and *Aspergillus* (Lai et al., 1991; Larsen and Breinholt,
256 1999; Malmstrom et al., 2000; Watanabe et al., 1998). The isolation of these compounds from
257 *Phoma* species is however novel. The biological activities of these isocoumarins area still
258 undetermined, but isocoumarins can have a wide range of biological activities; some are toxic to
259 animals including humans (Marquardt and Frohlich, 1992), whereas others have antimicrobial
260 (Okazaki et al., 1975), mutagenic (Varanda et al., 1997) or antitumor properties (Kuhr et al., 1973).
261 Citreo-isocoumarin and citreo-isocoumarinol were hypothesized as probable biosynthetic precursors

of citreviranol and demethylcitreoviranol (Lai et al., 1991), but it still unknown whether these compounds are produced by any of the *P. pomorum* strains or what role the isocoumarins play in the secondary metabolism of *P. pomorum*.

Previously, seven phomapyrones have been isolated from *P. lingam* (sexual stage *Leptosphaeria maculans*) (Pedras et al., 1994; Pedras and Chumala, 2005). We performed a preliminary search for $[M+H]^+$, $[M+NH_4]^+$ and [M+ CH3CN]$^+$ ions in *P. pomorum* extracts, as we did not have access to reference standards of these compounds. This search was however unsuccessful and it seems therefore unlikely that *P. pomorum* is a producer of these compounds.

Tenuazonic acid has previously been reported from extracts of a *P. sorghina* strain (Shephard et al., 1991), but neither the four *P. sorghina* strains used in the present study produced this compound, nor was it produced by any of the other species tested. However, Shephard et al. (1991) used autoclaved yellow corn kernels as growth medium for *P. sorghina*, which may indicate a medium dependent production of tenuazonic acid, if the compound is in fact is produced by *P. sorghina*.

The observation that *P. pomorum* is a solid producer of isocoumarins on artificial growth medium suggest that there is a risk that these metabolites may also be produced during maize infections in the fields. In a small pre-study we observed that *P. pomorum* strains produced isocoumarins on an artificial maize medium made from pieces derived from whole maize plants (unpublished data). On this medium diaportinic acid was the predominant analogue, although the levels were substantially lower than on DRYES. A LC-MS/MS method for detection of diaportinic acid was developed and used to screen maize samples derived from whole maize plants collected at harvest. We were however not able to detect to diaportinic acid in any of the examined samples, which indicates that the compound may not pose a risk to consumers of Danish maize products.

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393 **Figure 1.**

394 Fifty percent majority rule consensus tree from a BI analysis of ITS sequences of *Phoma* species
395 morphologically similar to *P. pomorum*. At the nodes the BI Posterior Probabilities are presented
396 above the branch, and bootstrap percentages of the ML analysis are given below the branch.
397 Branches that were less than 50% supported in the NJ and ML analyses are indicated with a hyphen.
398 The bar indicates the number of substitutions per site. The tree is rooted with *Phoma sorghina*.

399

400 **Figure 2.** Dendrogram from an automated Chemical Image Analysis (CIA) of 65 *Phoma* extracts
401 with the Ward clustering method. The scale (similarity) is arbitrary.

402

403 **Figure 3.** HPLC-UV-VIS chromatograms of selected *Phoma* and *Epicoccum* strains at 246 nm.
404 Peaks of citreo-isocoumarinol (I), citreo-isocoumarin (II), diaportinic acid (III), diaporthin (IV) and
405 dichlorodiaporthin (V) are indicated. The extracted spectrum of diaportinic acid has been inserted.

406

407 **Figure 4.** Structures of some isocoumarins produced by *P. pomorum*.

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Table 1. *Phoma and Epicoccum* strains used in the experiments.

#	Species	Source	Origin	IBT[a]	Other Collections[b]	GenBank[c]
1	*Phoma pomorum*	Maize	Denmark	41376	JLS P1	FJ839846
2	*P. pomorum*	Maize	Denmark	41453	JLS P2	FJ839855
3	*P. pomorum*	Maize	Denmark	41454	JLS P3	FJ839861
4	*P. pomorum*	Maize	Denmark	41377	JLS P4	FJ839862
5	*P. pomorum*	Maize	Denmark	41455	JLS P5	FJ839863
6	*P. pomorum*	Maize	Denmark	41456	JLS P6	FJ839865
7	*P. pomorum*	Maize	Denmark	41457	JLS P8	FJ839866
8	*P. pomorum*	Maize	Denmark	41458	JLS P9	FJ839867
9	*P. pomorum*	Maize	Denmark	41459	JLS P10	FJ839847
10	*P. pomorum*	Maize	Denmark	41460	JLS P11	FJ839848
11	*P. pomorum*	Maize	Denmark	41461	JLS P12	FJ839849
12	*P. pomorum*	Maize	Denmark	41462	JLS P13	FJ839850
13	*P. pomorum*	Maize	Denmark	41378	JLS P14	FJ839851
14	*P. pomorum*	Maize	Denmark	41463	JLS P15	FJ839852
15	*P. pomorum*	Maize	Denmark	41464	JLS P16	FJ839853
16	*P. pomorum*	Maize	Denmark	41465	JLS P18	FJ839854
17	*P. pomorum*	Maize	Denmark	41466	JLS P20	FJ839856
18	*P. pomorum*	Maize	Denmark	41467	JLS P21	FJ839857
19	*P. pomorum*	Maize	Denmark	41468	JLS P22	FJ839858
20	*P. pomorum*	Maize	Denmark	41469	JLS P24	FJ839859
21	*P. pomorum*	Maize	Denmark	41470	JLS P26	FJ839860
22	*P. pomorum*	Maize	Denmark	41476	JLS P51	FJ839864
23	*P. pomorum*	Gooseberry	Netherlands	41453	PD 81/592	FJ427057
24	*P. pomorum*	Bean	Netherlands	41485	PD 81/780	FJ839868
25	*P. pomorum*	Tartar Buckwheat	Netherlands		CBS 539.66	FJ427056
26	*P. pomorum*	Tomato	Netherlands	41495	CBS 115.67	FJ839840
27	*P. pomorum*	Barley	Germany	41555	CBS 838.84	FJ839845
28	*P. pomorum* var. *cyanea*	Wheat	South Africa	41505	CBS 388.80	FJ427055
29	*E. nigrum*[d]	Maize	Denmark	41380	JLS P28	
30	*E. nigrum*[d]	Maize	Denmark	41381	JLS P30	
31	*E. nigrum*[d]	Maize	Denmark	41472	JLS P31	
32	*E. nigrum*[d]	Maize	Denmark	41382	JLS P32	
33	*E. nigrum*	Man	Netherlands	41506	CBS 125.82	FJ426995
34	*E. nigrum*	soil	Germany	41510	CBS 505.85	FJ426997
35	*P. americana*	Maize	USA	41478	PD 80/1143	FJ426979
36	*P. americana*	-	-	41479	IMI 361195	FJ426976
37	*P. americana*	Sorghum	Nigeria	41482	PD 79/58	FJ426978
38	*P. americana*	Maize	South Africa	41484	PD 78/1089	FJ426977
39	*P. americana*	Maize	USA	41509	CBS 185.85	FJ426972
40	*P. americana*	Soybean	USA	41512	CBS 568.97	FJ426973
41	*P. americana*	Wheat	Argentina	41513	CBS 112525	FJ426975

42	*P. eupyrena*	soil	Germany	41487	CBS 527.66	FJ427000
43	*P. eupyrena*	Wheat	Germany	41507	CBS 832.84	FJ83944
44	*P. gardeniae*	Gardenia	India	41498	CBS 626.68	FJ427003
45	*P. gardeniae*	air	Netherlands Antilles	41514	CBS 302.79	FJ427002
46	*P. glomerata*	air	Netherlands	41480	PD 74/1023	FJ427018
47	*P. glomerata*	Ribus	Netherlands	41481	PD 73/1415	FJ427017
48	*P. glomerata*	Capsicum	-	41483	PD 83/782	FJ427021
49	*P. glomerata*	Potato	Germany	41489	CBS 293.36	FJ427010
50	*P. glomerata*	Tomato	Netherlands	41490	CBS 304.49	FJ427011
51	*P. glomerata*	fresco in church	Romania	41500	CBS 133.72	FJ427004
52	*P. glomerata*	Raspberry	Russia	41503	CBS 287.76	FJ427006
53	*P. glomerata*	bathroom	Netherlands	41511	CBS 464.97	FJ427012
54	*P. multirostrata*	Lily	-	41488	CBS 380.67	FJ427032
55	*P. multirostrata*	soil	India	41491	CBS 274.60	FJ427031
56	*P. multirostrata*	-	India	41492	CBS 340.65	FJ839843
57	*P. multirostrata*	soil	India	41493	CBS 368.65	FJ427033
58	*P. multirostrata*	Mango	Mali	41496	CBS 149.67	FJ839841
59	*P. multirostrata*	Sesame	India	41502	CBS 150.73	FJ839842
60	*P. sorghina*	Pine	USA	41477	PD 81/721	FJ427077
61	*P. sorghina*	Panicum miliare	India	41501	CBS 293.72	FJ427070
62	*P. sorghina*	Sorghum	Puerto Rico	41554	CBS 179.80	FJ427067
63	*P. sorghina*	Oat	Guinea-Bissau	41504	CBS 181.80	FJ427069
64	*P. zantedeschiae*	Calla lily	New Zealand	41486	CBS 131.93	FJ427084
65	*P. zeae-maydis*	Maize	USA	41499	CBS 588.69	FJ427086

[a] IBT collection at Department of Systems Biology, DTU, Denmark.
[b] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI: International Mycological Institute, Kew, UK; JLS: Culture collection of Jens Laurids Sørensen, housed at DTU; PD: Plant Protection Service, Wageningen, the Netherlands.
[c] Accession numbers of ITS sequences in GenBank.
[d] Not included in DNA sequence analysis.

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Table 2. Detection of some isocoumarins by HPLC-UV produced on DRYES by the various species.

	RI	$[M+H]^+$	*P. pomorum* (27)	*P. pomorum* var. *cyanea* (1)	*E. nigrum* (6)	*P. americana* (7)	*P. eupyrena* (2)	*P. gardeniae* (2)	*P. glomerata* (8)	*P. multistrata* (6)	*P. sorghina* (4)	*P. zantedeschiae* (1)	*P. zeae-maydis* (1)
Citreo-isocoumarinol [a]	739	281.10	27	0	0	0	0	0	2	0	0	0	0
Citreo-isocoumarin	744	279.08	27	0	0	0	0	0	2	0	0	0	0
Unknown [b]	757	283.08	11	1	0	6	1	0	5	0	0	1	1
Diaportinic acid	797	281.06	27	0	0	0	0	0	3	0	0	0	0
Diaporthin	859	251.09	22	0	0	0	0	0	0	0	0	0	0
Dichlorodiaporthin	993	319.01	27	0	0	0	0	0	3	0	0	0	0

[a] Tentatively identified

[b] Unidentified isocoumarin analogue, $[M+H]^+$ derived from fungal extracts.

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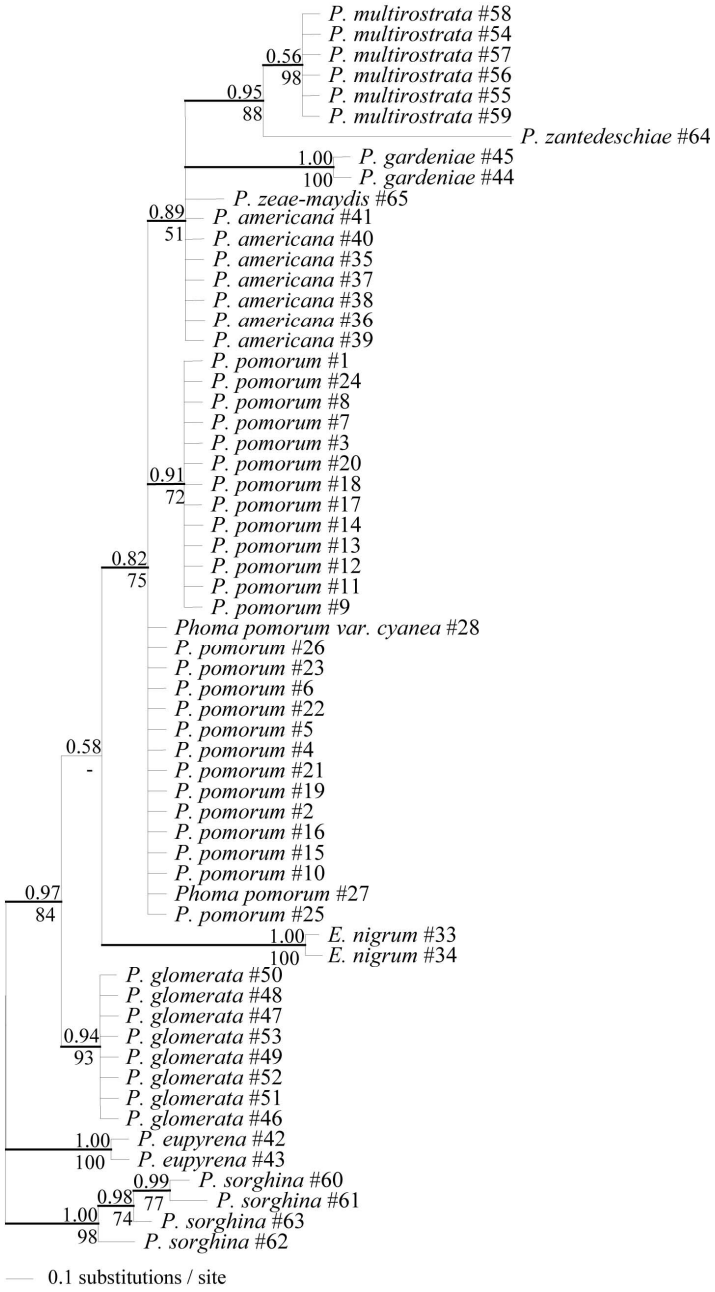
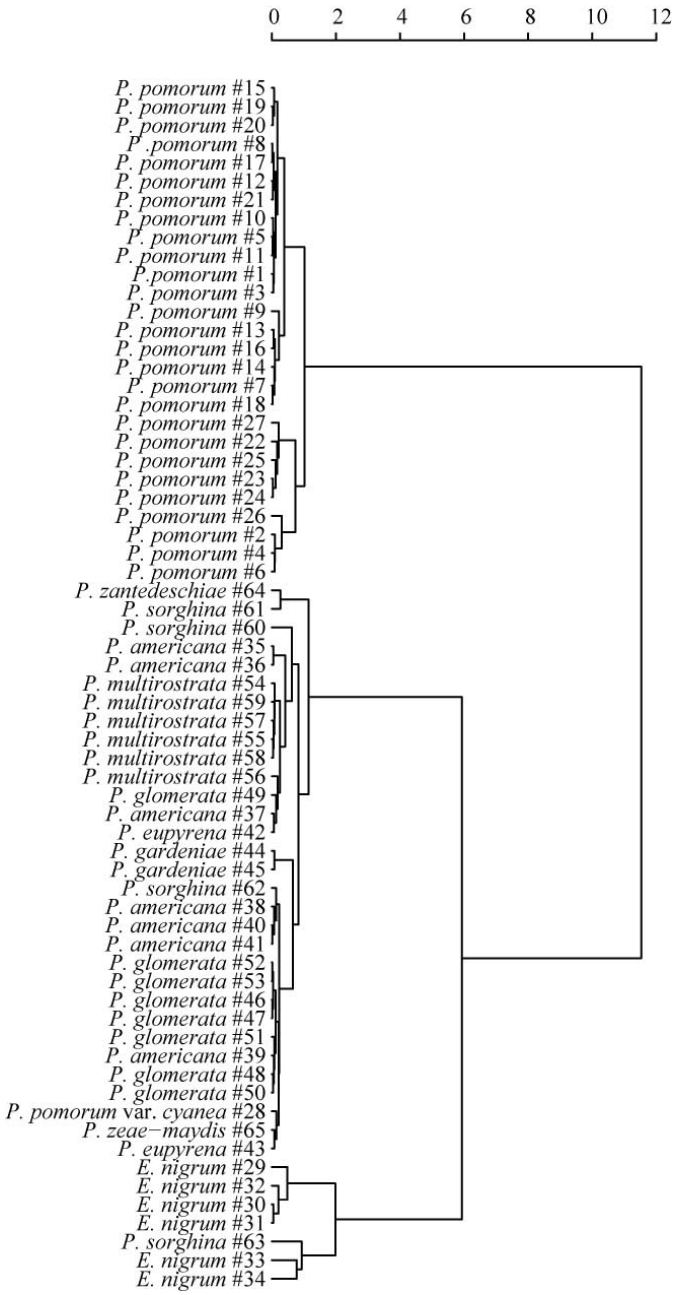
Figure 1


P. multirostrata #58
P. multirostrata #54
0.56
98
P. multirostrata #57
P. multirostrata #56
0.95
88
P. multirostrata #55
P. multirostrata #59
P. zantedeschiae #64
1.00
P. gardeniae #45
100
P. gardeniae #44
P. zeae-maydis #65
0.89
P. americana #41
51
P. americana #40
P. americana #35
P. americana #37
P. americana #38
P. americana #36
P. americana #39
P. pomorum #1
P. pomorum #24
P. pomorum #8
P. pomorum #7
P. pomorum #3
P. pomorum #20
0.91
P. pomorum #18
72
P. pomorum #17
P. pomorum #14
P. pomorum #13
0.82
P. pomorum #12
75
P. pomorum #11
P. pomorum #9
Phoma pomorum var. cyanea #28
P. pomorum #26
P. pomorum #23
P. pomorum #6
P. pomorum #22
P. pomorum #5
0.58
P. pomorum #4
-
P. pomorum #21
P. pomorum #19
P. pomorum #2
P. pomorum #16
P. pomorum #15
P. pomorum #10
0.97
Phoma pomorum #27
84
P. pomorum #25
1.00
E. nigrum #33
100
E. nigrum #34
P. glomerata #50
P. glomerata #48
P. glomerata #47
0.94
P. glomerata #53
93
P. glomerata #49
P. glomerata #52
P. glomerata #51
P. glomerata #46
1.00
P. eupyrena #42
100
P. eupyrena #43
0.99
P. sorghina #60
0.98
77
P. sorghina #61
1.00
74
P. sorghina #63
98
P. sorghina #62
0.1 substitutions / site

420 **Figure 2**



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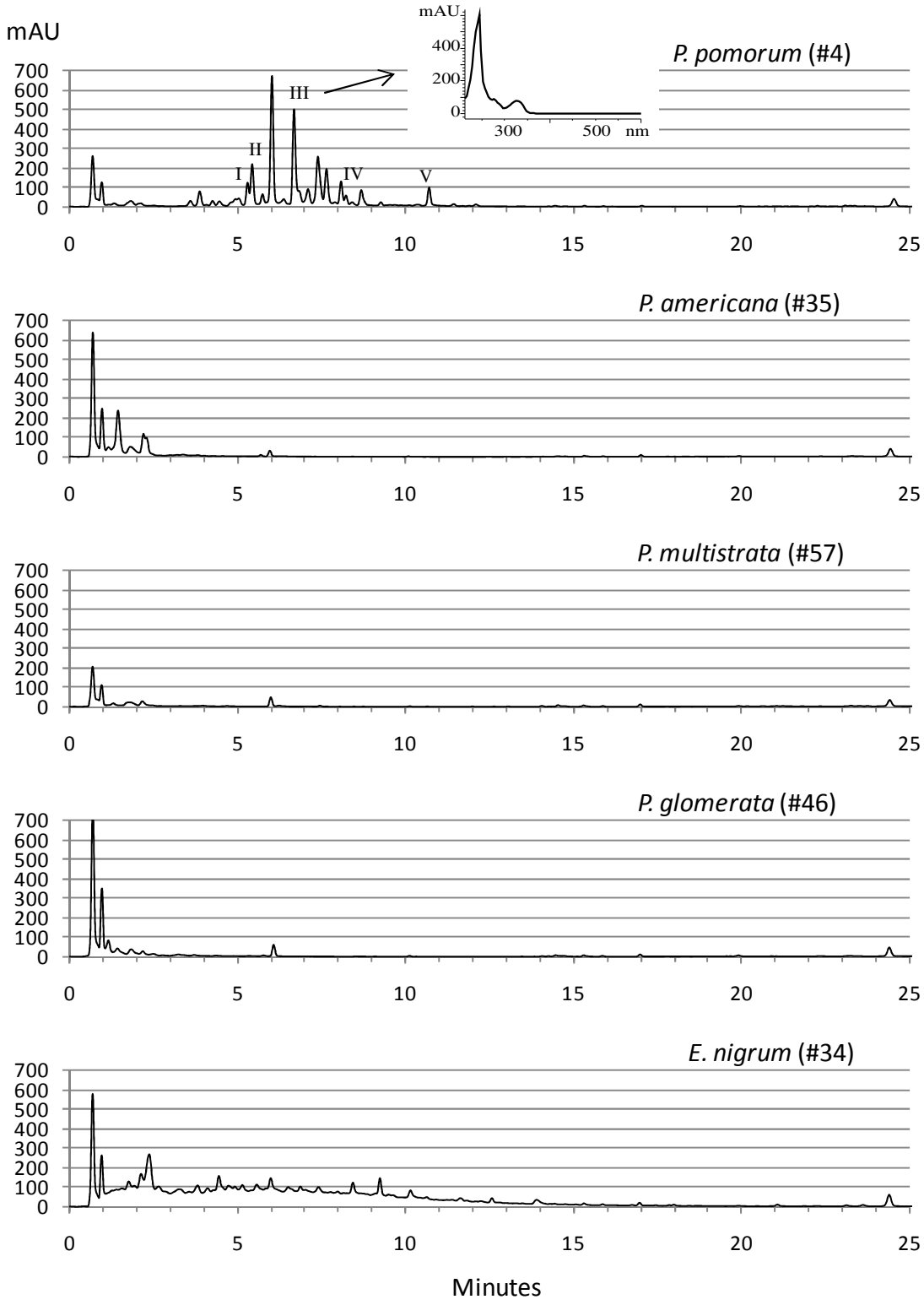
423 **Figure 3**



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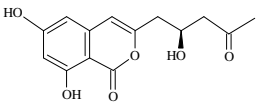
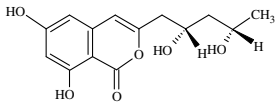
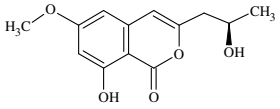
426 **Figure 4**



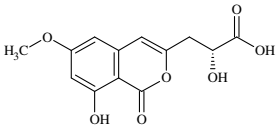
Citreo-isocoumarin



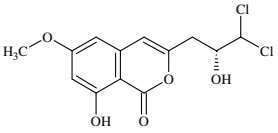
Citreo-isocoumarinol



Diaporthin



Diaportinic acid



dichlorodiaportin

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ORIGINAL MANUSCRIPT (VII)

Sørensen, J.L., Phipps, R.K., Nielsen, K.F., Frank, J., Schroers, H.J., and Thrane, U. (2009) Analysis of *Fusarium avenaceum* metabolites produced during wet apple core rot. *Journal of Agricultural and Food Chemistry* **57:** 1632-1639.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of *Fusarium avenaceum* Metabolites Produced during Wet Apple Core Rot

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Wet apple core rot (wACR) is a well-known disease of susceptible apple cultivars such as Gloster, Jona Gold, and Fuji. Investigations in apple orchards in Slovenia identified *Fusarium avenaceum*, a known producer of several mycotoxins, as the predominant causal agent of this disease. A LC-MS/MS method was developed for the simultaneous detection of thirteen *F. avenaceum* metabolites including moniliformin, acuminatopyrone, chrysogine, chlamydosporol, antibiotic Y, 2-amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol), aurofusarin, and enniatins A, A1, B, B1, B2, and B3 from artificially and naturally infected apples. Levels of moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 were quantitatively examined in artificially inoculated and naturally infected apples, whereas the remaining metabolites were qualitatively detected. Metabolite production was examined in artificially inoculated apples after 3, 7, 14, and 21 days of incubation. Most metabolites were detected after 3 or 7 days and reached significantly high levels within 14 or 21 days. The highest levels of moniliformin, antibiotic Y, aurofusarin, and the combined sum of enniatins A, A1, B, and B1 were 7.3, 5.7, 152, and 12.7 μg g^{-1}, respectively. Seventeen of twenty naturally infected apples with wACR symptoms contained one or more of the metabolites. Fourteen of these apples contained moniliformin, antibiotic Y, aurofusarin, and enniatins in levels up to 2.9, 51, 167, and 3.9 μg g^{-1}, respectively. Acuminatopyrone, chrysogine, chlamydosporol, and 2-AOD-3-ol were detected in 4, 11, 4, and 10 apples, respectively. During wet apple core rot, *F. avenaceum* produced high amounts of mycotoxins, which may pose a risk for consumers of apple or processed apple products.

KEYWORDS: Fusarium; mycotoxins; LC-MS-MS

INTRODUCTION

Fusarium avenaceum is one of the most commonly encountered *Fusarium* species in cereals and maize in northern Europe (*1*−*3*). During investigations in 2004−2006 in apple orchards in Slovenia, *F. avenaceum* was also identified as the causal agent of a wet apple core rot (wACR) (*4*). The disease is characterized by a white, rose, or reddish mycelium developing initially in the apple core and a light-brown wet rot spreading destructively into the surrounding cortex of infected apples (**Figure 1**). When harvesting the full yield of 21 Gloster apple trees in fall 2004, ca. 5% of the apples showed symptoms of wACR, and wACRs were also regularly encountered in other cultivars, such as Fuji and Jona Gold in 2004−2006 (*4*). The disease represents a potential economical problem for apple growers, who have to discard infected apples, and a safety problem for consumers due to potential production of mycotoxins during infections, especially for apple juice and cider production, since apples with infected cores can be removed from processes only with difficulties. *Fusarium avenaceum* is able to produce a wide range of chemically different bioactive secondary metabolites on artificial laboratory media (*5*), ranging from small polar compounds such as moniliformin and butenolide to larger apolar compounds such as enniatins (**Figure 2**). The toxicity of several *F. avenaceum* metabolites has been thoroughly investigated. The toxic mode of action of moniliformin is suggested to be linked with inhibition of enzyme systems and glucogenesis (*6*), and it can, like butenolide, induce myocardial damage (*7, 8*). Chlamydosporol and acuminatopyrone are related compounds of which the former has been shown to cause weight loss in rats and cytotoxicity in human and mouse cell lines (*9*). 2-Amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol) is a sphingosine analogue recently isolated from *F. avenaceum* (*10*). The compound is cytotoxic because it disrupts sphingolipid biosynthesis according to hypothesized models (*10*). Chrysogine, whose toxicity is undetermined, is produced by various phylogenetically unrelated ascomycetous genera including *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* (*11*−*14*). Fusarin A and C are metabolites produced by several *Fusarium* species (*15, 16*) and show mutagenic properties (*17*). Antibiotic Y was originally

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Figure 1. Early (top) and late (bottom) symptoms of wet apple core rot.

isolated from *F. lateritium* (*18*) and called lateropyrone. The structure was later amended and named antibiotic Y (*19*). This compound is produced by several *Fusarium* species, including *F. avenaceum*, *F. lateritium*, *F. torulosum, F. acuminatum*, *F. flocciferum*, and *F. tricinctum* (*18, 20*−*22*), and it has, as the name suggests, antibiotic properties (*18*). Aurofusarin is a red pigment belonging to the naphthoquinone group and is produced by several *Fusarium* species (*21, 22*). Aurofusarin has been shown to have a negative effect on the antioxidant system of the quail egg yolk and causes alterations in fatty acid composition of the egg yolk (*23*). Enniatins are cyclic hexadepsipeptides consisting of three alternating *N*-methyl-L-amino acid and D-α-hydroxyisovaleric acid residues, which are able to form cation selective channels in cellular membranes (*24*). They are cytotoxic (*5*) and toxic to insects (*25*), bacteria (*24*), and fungi (*26*). *Fusarium avenaceum* is able to produce at least six enniatins, which often occur in the following successively decreasing amounts in the sequence B > B1 > A1 > A > B2 > B3 on laboratory media (*5*). The enniatins occur in similar proportional amounts in Scandinavian cereals and maize, where *F. avenaceum* is one of the most abundant species (*27*−*29*). The bioactivity and natural occurrence have only been examined for a few metabolites, but fractionated extracts indicated in cytotoxicity tests that 2-AOD-3-ol and enniatins were the most potent cytotoxic compounds produced by *F. avenaceum (5).*

Analysis of naturally occurring *F. avenaceum* metabolites has mainly focused on moniliformin and enniatins A, A1, B, and B1 in cereal and maize based food and feeds. These compounds, together with antibiotic Y, were recently included in a LC-MS/MS screening method for 87 metabolites (*30*). In the present study, we developed a method for the simultaneous detection of thirteen *F. avenaceum* metabolites from apples collected in the field and showing a wet core rot and artificially induced apple core rots in healthy apples infected with spores of *F. avenaceum*.

MATERIALS AND METHODS

Chemicals and Standards. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were gradient grade, and other chemicals were analytical grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA). Moniliformin was purchased as the sodium salt from Sigma-Aldrich, and a 73 μg/mL stock solution was made in 85% MeCN (*3*). An enniatin reference standard was kindly provided by Dr. Rainer Zocher, Technical University of Berlin, Germany, consisting of 17% enniatin A, 34% enniatin A1, 24% enniatin B, and 26% enniatin B1 (distribution determined by HPLC-UV at 200 nm and validated by LC-HRMS) (*28*). Of this solution, a 400 μg/mL stock solution was made in 100% MeCN and stored at −20 °C.

Antibiotic Y and chrysogine were available from previous studies (*31*), and a 250 μg/mL stock solution was made of the first in 100% MeCN and stored at −20 °C. 2-Amino-14,16-dimethyloctadecan-3-ol was a kind gift from Dr. Silvio Uhlig, National Veterinary Institute, Norway. A 250 μg/mL (100% MeCN) stock solution of aurofusarin was prepared from *F. graminearum* as described below and stored at −20 °C.

Aurofusarin Standard. Aurofusarin was extracted from *F. graminearum* (IBT 41393, available at the author's address) grown for 14 days at 25 °C in the dark on 9 cm (ID) millet agar plates (30 g of organic millet grains, autoclaved in 1000 mL of distilled H_2O, 15 g of agar, and 1 mL of trace metal solution consisting of 10 g/L $ZnSO_4 \cdot 7H_2O$ and 5 g/L $CuSO_4 \cdot 5H_2O$). The aerial mycelium of 400 plates were scraped off and extracted multiple times with ethyl acetate and finally acidic ethyl acetate while mixing using an Ultra Turrax T25 basic (IKA Werke GmbH & Co. KG, Staufen, Germany). The pooled extracts were evaporated to dryness *in vacuo*, dissolved in 50 mL of methanol, and filtered though a Whatman no. 4 filter (Brentford, U.K.). Primary separation was done on a Sephadex LH20 column (1000 mm × 50 mm, flow rate: 5 mL/min), eluting with methanol and collecting 15 mL fractions. The fractions were combined to 10 portions based on color. The fifth portion contained both aurofusarin and zearalenone and was subjected to multiple LC runs on a Gemini C_6-Phenyl (150 mm × 10 mm i.d., 5 μm) column (Phenomenex, Torrance, CA) using a H_2O−MeCN gradient system (20 mL/min) starting at 15% MeCN and increasing to 100% over 20 min, with aurofusarin eluting at 9.7 min. Analysis of a ^1H NMR spectrum in CD_3OD [Bruker 500 MHz] showed four singlets signals (methyl, methoxy, vinyl, and aromatic) matching data in ref *32*, and integrals showed a purity of >95%, which was also confirmed by LC-DAD-HRMS (*31*).

Fungal Strains, Media, and Growth Conditions. Six *F. avenaceum* strains isolated from maize and five strains from apples (**Table 1**) were grown on potato dextrose agar (PDA) (*21*), yeast extract agar (YES) (*21*), and apple agar (AA) to determine the metabolite profile of *F. avenaceum* on artificial growth media. AA with a pH value of 3.3 was made by autoclaving 300 g of homogenized Gloster apples (obtained from a local Danish supermarket) and distilled H_2O, 1 mL of a trace metal solution (10 g/L $ZnSO_4 \cdot 7H_2O$ and 5 g/L $CuSO_4 \cdot 5H_2O$ in a 1000 mL volume), and 30 g of agar. All media were cooled to 45 °C and poured into sterile 90 mm Petri dishes. The 11 *F. avenaceum* strains were transferred aseptically to the agar plates in three point inoculations and incubated 14 days at 25 °C in the dark.

Extraction of Metabolites from *F. avenaceum* Grown on PDA, YES, and AA. A modified version of the previously described microscale extraction method (*33*) was used. In brief, 9 agar plugs were taken from the colony center of cultures 14 days old and extracted ultrasonically for 45 min with 1.5 mL of ethyl acetate (0.5% formic acid) and subsequently with 1.5 mL of isopropanol. The solvent was removed *in vacuo*, redissolved in 400 μL of methanol, filtered through a 0.45 μm PTFE syringe filter (National Scientific, Rockwood, TN), and analyzed.

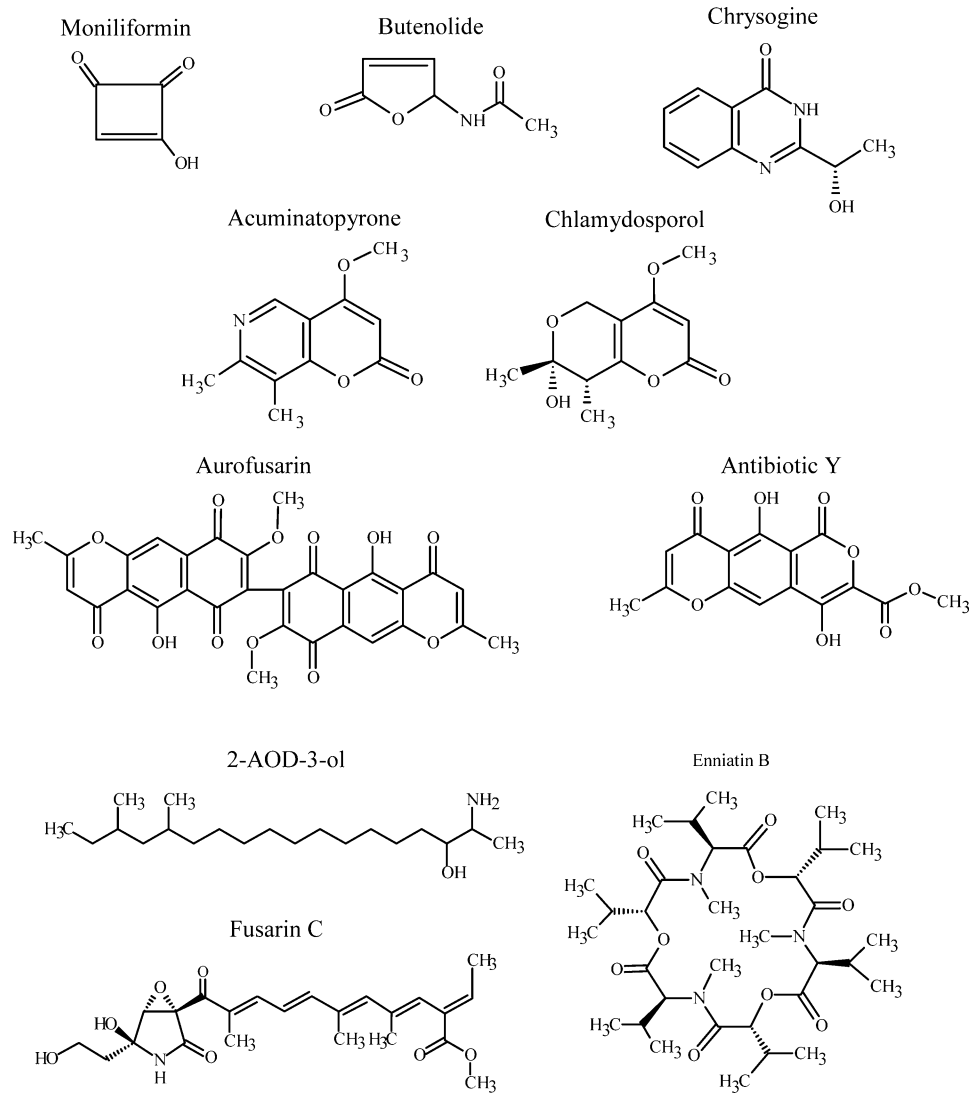

Figure 2. Metabolites potentially produced by *F. avenaceum*.

Table 1. *Fusarium avenaceum* Strains from Denmark (DK) and Slovenia (SI) Used in Experiments

strains[a]	origin	IBT collection[b]
JLS F1[c]	maize, DK	IBT 41177
JLS F2[c]	maize, DK	
JLS F3[c]	maize, DK	
JLS F4[c]	maize, DK	
JLS F29[c]	maize, DK	
JLS F35[c]	maize, DK	IBT 41180
HJS 346[c]	apple, SI	IBT 41123
HJS 846[c]	apple, SI	IBT 41117
HJS 848[c,d]	apple, SI	IBT 41118
HJS 849[c]	apple, SI	IBT 41122
HJS 851[c,d]	apple, SI	IBT 41119
HJS 288[d]	apple, SI	IBT 41125
HJS 911[d]	apple, SI	
HJS 912[d]	apple, SI	

[a] Number in personal collections of J. L. Sørensen (J.L.S.) and H.-J. Schroers (H.J.S.). [b] Number in IBT collection, Technical University of Denmark, Department of Systems Biology. [c] Strains used to determine metabolites from artificial culture media. [d] Strains used to artificially inoculate apples.

Spiked Apples. Material from a *Fusarium* free Golden Delicious apple, which did not contain any *Fusarium avenaceum* metabolites, was used to spike with moniliformin, antibiotic Y, aurofusarin, and enniatins. Freeze-dried apple material, equivalent to 30 g of fresh apple, was extracted ultrasonically for 60 min with 150 mL of MeCN−H_2O (85:15). One milliliter subextracts were spiked with moniliformin, antibiotic Y, aurofusarin, and enniatins to obtain the levels shown in **Table 2**.

Table 2. Spike Levels of Moniliformin, Antibiotic Y, Aurofusarin, and Enniatins (ng g^{-1})[a]

moniliformin	0	50	100	200	400	800	1600	3200	6400	12800
antibiotic Y	0	50	100	200	400	800	1600	3200	6400	12800
aurofusarin	0	50	100	200	400	800	1600	3200	6400	12800
enniatin A	0	17	34	67	134	268	537	1073	2146	4292
enniatin A1	0	34	67	134	269	537	1075	2149	4298	8596
enniatin B	0	24	48	95	190	380	760	1520	3040	6080
enniatin B1	0	26	52	104	207	414	829	1658	3316	6632

[a] Samples were spiked in triplicate.

Artificial Inoculation of Apples. Healthy apple fruits of cultivar Golden Delicious were obtained from a local supermarket in Slovenia. The calyx region and the upper parts of the apples were wetted with ethanol (96%) and flambéed for several seconds. The apples were then artificially infected with 100 μL of a conidial suspension (ca. 10^6/mL) of selected *F. avenaceum* strains (**Table 1**). The conidial suspensions were prepared from 21 day old SNA/C cultures (Spezieller Nährstoffarmer Agar (*34*) amended with 50 g/L of finely sliced carrot pieces and filtered through three layered cheesecloth). The conidial suspension was injected with a sterile syringe and injection needle (0.8 mm × 38 mm), which was moved through the calyx region into the apple cores. Apples were incubated for 3, 7, 14, and 21 days at 22−24 °C and

Table 3. Parameters Used in the Mass Spectrometries Including Ionization Mode (mode), Retention Time (RT/min), Scan Range (range/min), Transition Ions (ion/*m*/*z*), Cone (v), and Collision Energy (CE/V)

compd	mode	RT	range	ion[a]	ion[b]	ratio[c]	cone	CE
moniliformin	−	3.0	1.0−4.5	Qt	97.0 → 41.2		25	15
acuminatopyrone	+	5.3	3.5−6.5	Qt	206.1 → 106.2	1.5	40	35
				Ql	206.1 → 174.0			25
chrysogine	+	6.8	4.5−8.0	Qt	191.1 → 130.1	1.6	20	30
				Ql	191.1 → 155.1			30
chlamydosporol	+	7.5	6.0−9.0	Qt	227.1 → 167.1	17.8	30	35
				Ql	227.1 → 107.2			20
antibiotic Y	+	11.3	10.0−12.5	Qt	319.0 → 286.9	491	20	15
				Ql	319.0 → 175.1			15
2-AOD-3-ol	+	11.8	10.5−13.2	Qt	314.3 → 296.3	131	20	15
				Ql	314.3 → 111.3			15
aurofusarin	+	13.3	12.0−14.5	Qt	571.1 → 484.8	1.2	60	60
				Ql	571.1 → 456.8			50
enniatin B3	+	15.0	14.0−15.8	Qt	612.4 → 196.1	1.8	100	25
				Ql	612.4 → 214.1			25
enniatin B2	+	15.3	14.5−16.5	Qt	626.4 → 196.1	1.7	100	25
				Ql	626.4 → 214.1			25
enniatin B	+	15.8	15.0−17.0	Qt	640.4 → 196.1	15.8	100	25
				Ql	640.4 → 527.3			25
enniatin B1	+	16.2	15.4−17.4	Qt	654.4 → 196.1	2.6	100	25
				Ql	654.4 → 228.0			25
enniatin A1	+	16.8	15.8−17.8	Qt	668.4 → 210.2	4.0	100	25
				Ql	668.4 → 541.2			25
enniatin A	+	17.2	16.2−18.2	Qt	682.4 → 210.1	4.6	100	25
				Ql	682.4 → 555.0			25

[a] Qt: quantifier, Ql: qualifier. [b] All transitions were made from $[M + H]^+$ except for moniliformin, which was made from $[M - H]^-$. [c] Ratio of quantifier and qualifier ions was calculated from spiked samples and media extracts.

subsequently cut open longitudinally. Rotten parts of the core and cortex were harvested with a spoon, weighed, ground with a mortar and pestle in liquid nitrogen, and freeze-dried.

Naturally Infected Apples. Twenty naturally infected Gloster apples showing wet apple core rot symptoms were obtained from trees in the experimental orchard of the Agricultural Institute of Slovenia, Brdo pri Lukovici, near harvest time. Each apple was processed for metabolite detection individually as described above.

Extraction of Metabolites from Apples. Freeze-dried apple material, equivalent to 2 g of fresh weight, was extracted ultrasonically for 60 min with 10 mL of MeCN−H_2O (85:15). Subsamples (1 mL) were filtered through 0.45 μm PTFE syringe filters and analyzed.

LC-MS/MS. Liquid chromatography was performed on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany). Eight microliters of extract was injected and separated on a Gemini C_6-Phenyl 3 μm 2-mm i.d. × 50-mm column (Phenomenex, Torrance, CA) using a constant flow of a 0.3 mL/min MeCN−water gradient, starting at 0% MeCN and going to 100% over 21 min, followed by a 2 min wash with 100% MeCN at 0.5 mL/min before reverting to 0% MeCN over 1 min and maintaining for a further for 5 min. The water and MeCN were buffered with 20 mM formic acid. The LC was coupled to a Quattro Ultima triple mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray ESI source using a flow of 700 L/h nitrogen at 350 °C; hexapole 1 was held at 12 V. The system was controlled by MassLynx 4.1 (Waters-Micromass). Nitrogen was also used as collision gas, and the MS was operated in the multiple reaction monitoring (MRM) mode (dwell time 200 ms) with the parameters shown in **Table 3**. Chromatography and MS/MS were optimized on pure standards of moniliformin, chrysogine, antibiotic Y, 2-AOD-3-ol, aurofusarin, and enniatins A, A1, B, and B1. The settings for acuminatopyrone, chlamydosporol, and enniatins B2 and B3 were optimized from fungal extracts because standards were not available. The identities of these compounds in the fungal extracts were validated by LC-DAD-HRMS (*31*). All metabolites were analyzed in positive ionization mode with $[M + H]^+$ as the parent ion, except moniliformin, which was analyzed in negative ionization mode with $[M - H]^-$ as the parent ion. Collision energy was optimized for the two specific (no loss of H_2O, NH_3, CO_2) major fragment ions for each compound (**Table 3**). The limits of quantification (LOQ) for moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 were set as the minimum spike level (**Table 2**), which for quantifier ions had an average signal-to-noise (S/N) ratio of 13, 142, 8, 62, 80, 94, and 50, respectively. The limits of detection (LOD) were estimated to be linear down to S/N ratios of 5, resulting in LOD for moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 of 19, 2, 31, 1, 2, 1, and 3 ng g^{-1}, respectively. The LOQ for acuminatopyrone, chrysogine, chlamydosporol, and 2-AOD-3-ol was set to 0.1% of the maximum level detected.

RESULTS AND DISCUSSION

Method Development. Liquid chromatography was done with a Gemini C_6 phenyl column with a mobile solvent gradient starting at 100% H_2O in order to achieve satisfactory retention of moniliformin (RT: 3.0 min, $k' = 3.6$).

As a combined effect of the ability of the column to retain aromatic compounds and the low start concentration of organic solvent, chrysogine was also very well retained. The first target metabolite, moniliformin, eluted after 3.0 min, and the last, enniatin A, eluted after 17 min. In this window all other eleven metabolites eluted with little or no overlap (**Figure 3**). The good retention of moniliformin is also seen on a Gemini C_{18} column (*30*), indicating that the retention is probably caused by retention of the Gemini particles rather than the RP phase. When a Luna phenyl C_6 column (Phenomenex) with identical dimensions to the Gemini phenyl C_6 column was used with the same solvent gradient, moniliformin eluted after 1.7 min, further supporting that the good retention of moniliformin is due to the Gemini particles.

Butenolide could not be properly retained (RT: <1 min, $k' = 0.2$) and was therefore not included in the method, due to the risk of coelution with matrix components. Fusarins A and C were also not included in the method because they showed poor chromatography, resulting in multiple smaller peaks over 1−2 min.

Metabolite Production on Artificial Media. Eleven *F. avenaceum* strains were grown on PDA, YES, and AA to analyze the metabolite potential in a small scale experiment with the developed LC-MS/MS method (**Table 4**). All strains produced detectable amounts of moniliformin, chrysogine,

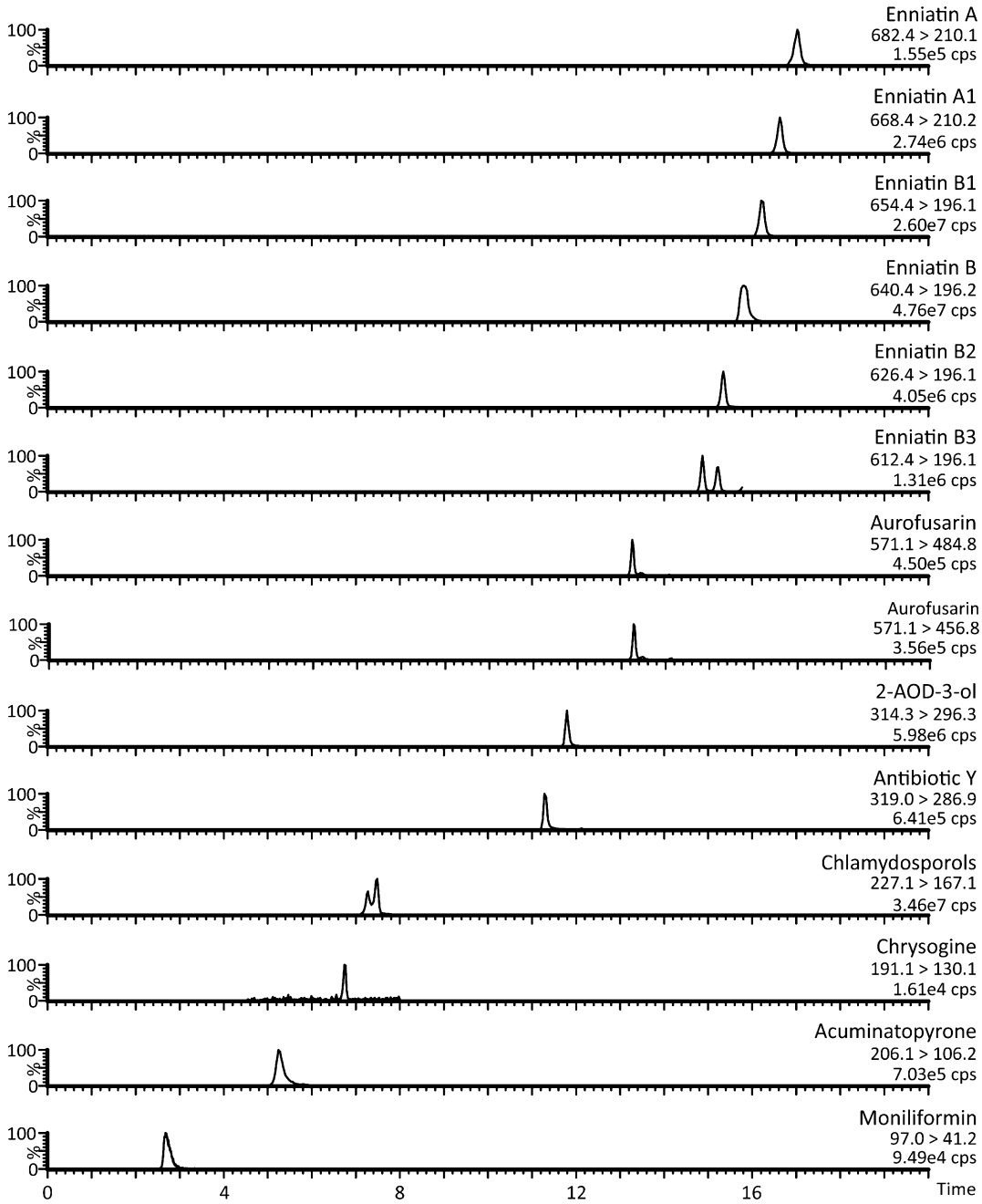

Figure 3. LC-MS/MS detection of quantifier ions of 13 metabolites from *F. avenaceum* strain JLS F29 on YES in counts per seconds (cps) and percent. Qualifier ion of aurofusarin is also included.

antibiotic Y, 2-AOD-3-ol, aurofusarin, and the six enniatins on all three media. Unexpectedly, two peaks were detected with the enniatin B3 settings. We suggest that the first peak is derived from enniatin B3, as this peak was from a compound eluting 0.4 min before enniatin B2, which is the same interval as between the other B type enniatins. The second peak might be from another enniatin with an identical mass and fragmentation pattern, which could be enniatin J1 (*35*). Two overlapping chlamydosporol peaks were detected from all strains on PDA and YES and from 6 strains on AA. This suggests the presence of chlamydosporol and one of its analogues (such as iso-chlamydosporol) having an identical mass and fragmentation pattern, which previously has also been observed in *F. tricinctum* and *F. chlamydosporum* (*36*). Distribution of acuminatopyrone production was sparse, as it was detected only in two strains on AA and PDA and four strains on YES. The distribution of the metabolites in this small study concurs with the large scale study made by Uhlig et al. (*5*). The acidic apple agar (pH 3.3) did not seem to inhibit production of any of the metabolites, and it is therefore likely that all metabolites can be present in apples infected with *F. avenaceum*.

Detection Limits and Recovery of Selected Metabolites from Spiked Apples. Apple samples were spiked with moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 in triplicate to obtain the 10 levels shown in **Table 2**. All metabolites were recovered linearly with high R^2 values ranging from 0.987 to 0.999 (**Table 5**). Little or no matrix effects were observed for moniliformin, antibiotic Y, and aurofusarin; however, the apple matrix seemed to enhance the enniatin signals, resulting in apparent recoveries above 100%, which we also have observed previously with maize matrix (*28*). The LOD of moniliformin was 19 ng g^{-1}, which is comparable to other LC-MS/MS methods that had 10 ng g^{-1} (*27*) and 20 ng g^{-1} (*30*). The LOD for the four enniatins was also in the range of previously published LC-MS/

Table 4. Production of Metabolites by 11 *Fusarium avenaceum* Strains on AA, PDA, and YES

	AA	PDA	YES
moniliformin	11	11	11
acuminatopyrone[a]	2	2	4
chrysogine	11	11	11[c]
chlamydosporols[b]	6	11	11
antibiotic Y	11	11	11
2-AOD-3-ol	11	11	11
aurofusarin	11	11	11
enniatin B3	11	11	11
enniatin B2	11	11	11
enniatin B	11	11	11
enniatin B1	11	11	11
enniatin A1	11	11	11
enniatin A	11	11	11

[a] By JLS F29 and HJS 846 on all three media; JLS F3 and F35 also on YES. [b] By JLS F29, F35, HJS 346, 846, 848, and 849 on AA. [c] Trace amount by HJS 346.

Table 5. Recovery of Moniliformin, Antibiotic Y, Aurofusarin, and Enniatins from Spiked Apples

metabolite	n[a]	spike level (ng g^{-1})	recovery % (min−max)	st dev[b]	R^2
moniliformin	27	50−12800	100 (89−112)	6	0.999
antibiotic Y	27	50−12800	93 (75−112)	11	0.998
aurofusarin	27	50−12800	101 (70−156)	22	0.987
enniatin A	27	17−4352	115 (98−149)	13	0.999
enniatin A1	27	34−8704	113 (99−141)	10	0.999
enniatin B	27	24−6144	113 (98−166)	17	0.997
enniatin B1	27	26−6566	114 (98−145)	14	0.999

[a] Number of samples. [b] Standard deviation.

MS methods. For instance, the LOD of enniatin B was 1 ng g^{-1} in our method, which is lower than we had in a previous study of maize (12 ng g^{-1}) (*28*), but higher than others who had 0.4 ng g^{-1} (*27*) and 0.03 ng g^{-1} (*30*). The spiked apple samples were used to calculate the occurrence of moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1.

Artificially Infected Apples. Five strains of *F. avenaceum* (**Table 1**) were used to artificially infect apples, and their metabolites after 3, 7, 14, and 21 days of incubation were determined. Strain HJS 912 did not produce detectable amounts of any of the target metabolites except for aurofusarin and 2-AOD-3-ol (data not shown). The remaining four strains produced detectable amounts of moniliformin, chrysogine, antibiotic Y, 2-AOD-3-ol, aurofusarin, and enniatins A, A1, B, and B1 except for strain HJS 911, which did not produce moniliformin (**Figure 4**).

Moniliformin was detected after 7 days, reaching maximum amounts after 14 days in strains HJS 288 (7.34 μg g^{-1}) and HJS 851 (1.29 μg g^{-1}) and after 21 days in HJS 848 (3.34 μg g^{-1}). Antibiotic Y was detected after 3 days in strain HJS 848, whereas it could be detected after 7 days in 288 and HJS 851 and after 21 days in strain HJS 912. Antibiotic Y levels were highest after 14 days in strain HJS 851 (0.21 μg g^{-1}) and 21 days in strains HJS 288 (0.26 μg g^{-1}), HJS 848 (5.7 μg g^{-1}), and HJS 912 (0.12 μg g^{-1}). Aurofusarin was detected after 3 days in all strains and reached a maximum after 14 days in strains HJS 848 (140 μg g^{-1}), HJS 851 (108 μg g^{-1}), and HJS 912 (152 μg g^{-1}) and after 21 days in strain HJS 288 (107 μg g^{-1}). These levels vastly exceeded the maximum spike level (12.8 μg g^{-1}), which is why accurate quantification in these samples is difficult. If present, the four enniatins, whose summed total is shown, occurred in successively decreasing amounts in the sequence B > B1 > A1 > A, as previously noticed (*5, 28*). Strains HJS 288 and HJS 848 produced the highest amounts of enniatins (12 μg g^{-1} and 13 μg g^{-1}, respectively) after 14 days and were detected after 3 and 7 days, respectively. Enniatins could only be detected in strain HJS 851 after 14 days (0.47 μg g^{-1}) and after 3 days in strain HJS 912 (trace). Enniatin B2 was detected in the five samples with the highest amounts of enniatins A, A1, B, and B1, whereas B3 was only detected in the top three samples (data not shown).

2-AOD-3-ol was produced by all four strains and was detected after 3 days in strains HJS 848, HJS 851, and HJS 912 and after 7 days in strain HJS 288. The level of the metabolite was highest after 14 days in strains HJS 288, HJS 848, and HJS 851 and after 21 days in 912. Chrysogine was detected after 7 days in strain HJS 851 and after 14 days in strains HJS 288, HJS 848, and HJS 912. Strains HJS 288, HJS 851, and HJS 912 produced maximum levels after 14 days and strain HJS 848 after 21 days. Acuminatopyrone and chlamydosporols could only be detected in samples inoculated with strain HJS 288 (data not shown).

In general, all strains produced significantly accumulated amounts of various metabolites after either 14 or 21 days. Interestingly, highest amounts of moniliformin, aurofusarin, 2-AOD-3-ol, and chrysogine were measured in the majority of samples already after 14 days, which may indicate that the metabolism rate of the strains decreases or comes to a steady state after a certain amount of time.

Naturally Infected Samples. Twenty apples showing symptoms of wACR were collected and analyzed. One or several *F. avenaceum* metabolites were detected in 17 samples (**Table 6**). Antibiotic Y and aurofusarin were the most frequently observed metabolites occurring in 16 samples in levels up to 51 μg g^{-1} and 145 μg g^{-1}, respectively. Moniliformin was found in 14 samples ranging from trace amounts to 2.9 μg g^{-1}. The moniliformin levels were further validated by HILIC-UV-MS (*3*) (results not shown). The same 14 samples also contained at least one of the four enniatins A, A1, B, and B1, and the summed amounts of the enniatins ranged from trace amounts to 3.9 μg g^{-1}. Enniatin B2 was also present in these samples whereas B3 was present in 8 samples. Enniatins B2 and B3 were not quantified, but they will most likely only give a small contribution to the total enniatin amounts, given that these two analogues are normally produced in much smaller quantities than the other four major enniatins A, A1, B, and B1.

Chrysogine was found in 10 and 2-AOD-3-ol in 11 samples. To our knowledge, this is the first report of 2-AOD-3-ol detected in a naturally infected sample, while it has been observed only in artificially inoculated wheat (*10*). We were unable to quantify the levels of 2-AOD-3-ol, however, as we did not have sufficient amounts to spike apple samples for standards. LC-UV analyses of the raw extracts could not be used for estimating its quantity because 2-AOD-3-ol is not UV active. Chlamydosporols and acuminatopyrone co-occurred in four samples, suggesting that these two structurally related compounds share the same regulation mechanism. A clear correlation between the quantities of the various compounds, however, was not obvious, confirming the results obtained from the artificially infected apples.

Results of the artificially inoculated and naturally infected apples showed that antibiotic Y and aurofusarin are the most abundant metabolites, both occurring in relatively high amounts. To our knowledge, aurofusarin contamination has only been quantified once before, which was in wheat with levels up till 4.2 μg g^{-1} (*37*). We detected aurofusarin in much higher amounts, probably because the rots in our samples were well-developed. Biosynthesis of pigments, including aurofusarin, has been identified as a response of fungi to major stress factors such as low or high pH (*32*), and the relatively low pH in apples may have triggered

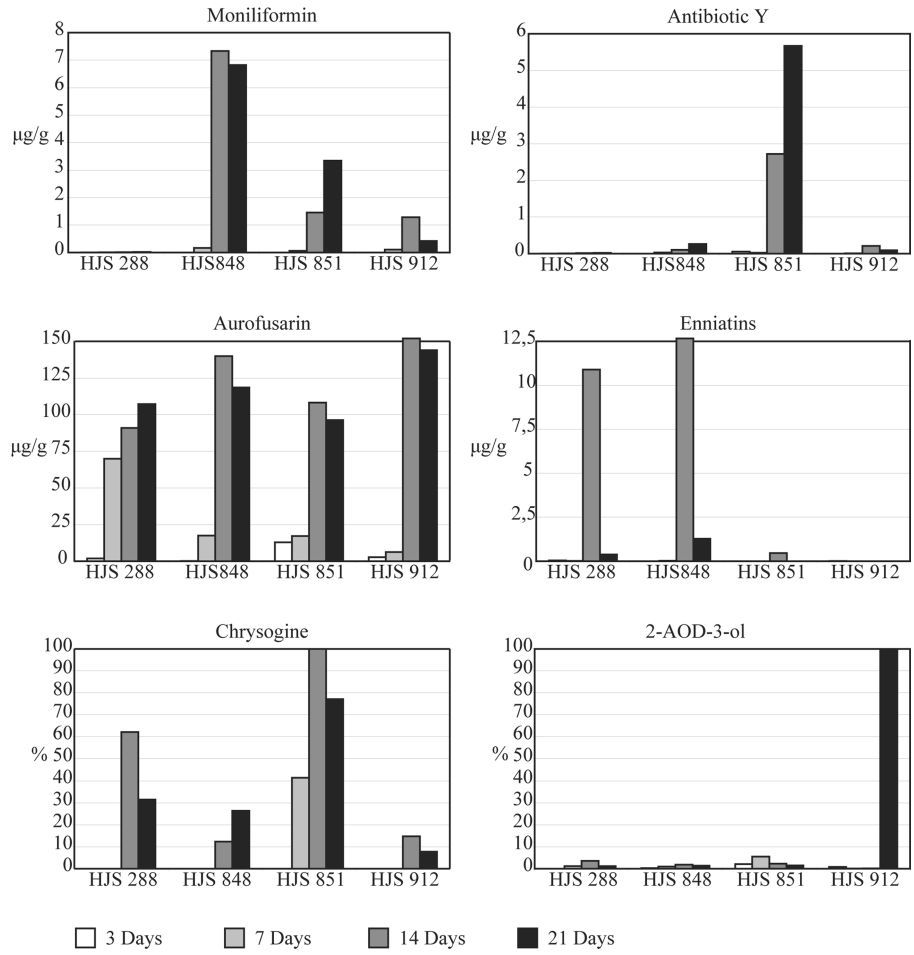

Figure 4. Occurrence of moniliformin, antibiotic Y, aurofusarin, and the sum of the four enniatins A, A1, B, and B1 (ng g^{-1}) in apples artificially inoculated with four *F. avenaceum* strains after 3, 7, 14, and 21 days of incubation. For 2-AOD-3-ol and chrysogine, the relative occurrence is given with the highest encountered amount set to 100.

Table 6. Occurrence of Moniliformin (mon), Antibiotic Y (anti Y), Aurofusarin (auro), and Combined Sum of Enniatins (enn) (μg/g) and 2-AOD-3-ol, Chrysogine (chrys), Chlamydosporols (chlam), and Acuminatopyrone (acumi) (%) in 20 Naturally Infected Apples Sampled from Trees[a]

	μg g^{-1}				%[b]			
#	mon	anti Y	auro	enn	2-AOD-3-ol	chrys	chlam	acumi
1	1.28		13.2	0.13	0.4	45.2	27.4	63.4
2								
3								
4	trace	2.57	0.64	0.34	0.4			
5	0.25	9.68	74.4	1.63	24.7	44.7		
6	2.87	trace	80.7	trace		12.9		
7	0.20	trace	145.1		4.3	11.3		
8	trace	0.29	34.4	0.12	2.1			
9		trace						
10	2.53	1.55	128.1	3.89	0.6	17.5		
11	2.74	4.17	166.6	0.54	24.0	3.0		
12	0.34	7.60	63.8	0.16	7.1	2.8	25.8	89.8
13		trace	0.18					
14	0.16	17.1	95.4	1.65	11.3	29.5	100	100
15	1.53	0.11	91.6	0.31	1.5	100		
16	0.33	0.08	1.20	0.20		25.4	62.6	3.2
17	1.32	51.3	103.6	2.60	100	21.0		
18	2.33	0.32	132.5	0.16	4.8			
19								
20								

[a] LOD and LOQ, see Material and Methods; trace, below LOQ (see Materials and Methods). [b] Highest peak area of each metabolite set to 100%.

production of high levels of aurofusarin in wet core rot samples. The toxicity of aurofusarin, however, is insufficiently known and it is therefore not possible to estimate its risk for apple consumers.

It should be noted that LC-MS/MS, due to is high selectivity, is blind for everything else and would thus overlook compounds not screened for. However, LC-DAD-HRMS (both ESI^+ and ESI^-) analysis of diluted extracts did not indicate other compounds in as high amounts as the selected target compounds. But clearly we would have overlooked compounds produced in low amounts and not expected from *F. avenaceum*.

The occurrence of several different types of mycotoxins produced by *F. avenaceum* is also a risk factor due to possible synergistic effects. As data on the cytotoxicity and mode of action of many of the mycotoxins produced by *F. avenaceum* are almost completely lacking, more effort in this area is needed for proper risk assessment (*38*). Because the infections start inside the apples where they cannot be seen, there is a high risk that mycotoxin contaminated apples would end up in food products such as apple juice, which again would have unacceptably high concentrations of mycotoxins. Thus, this disease could have devastating economic implications for apple orchards.

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TITLE:

Genetic diversity, mycotoxin production and pathogenicity of *Fusarium* spp. (*Gibberella fujikuroi* species complex) associated with rice seeds originating from Africa and Asia

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RUNNING TITLE

Characterisation of *Gibberella fujikuroi* from rice

KEY WORDS

Bakanae, *Gibberella fujikuroi* species complex, *Fusarium, F. fujikuroi, F. verticillioides, F. proliferatum, F. andiyazi*, rice, fumonisin, gibberellin, phylogeny

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SUMMARY

Strains of *Gibberella fujikuroi* species complex were isolated from rice (*Oryzae sativa* L.) seed originating from African and Asian countries. With exception of seeds originating from Bangladesh, the natural incidence of *G. fujikuroi* in rice seeds was high, ranging from 40% to 83%. Phylogenetic studies showed that there is a broad genetic variation among the strains, especially in the strains from China and Vietnam. *Fusarium verticillioides, F. fujikuroi, F. proliferatum* and *F. andiyazi* were commonly found associated with seeds of the different rice varieties. With the exception of *F. fujikuroi*, which was only detected in seed samples originating from Asia, all the four identified Fusaria were found in both African and Asian seed samples. Pathogenicity tests showed that all strains were able to reduce seed germination and possessed varying ability to cause Bakanae symptoms in rice; some species (i.e. *F. fujikuroi*) being more pathogenic than others. The ability to produce fumonisins (FB1 and FB2) and gibberellin A3 *in vitro* also differed according to the *Fusarium* species. While fumonisins were produced at significant levels (> 1µg/ml) by most of the strains of *F. verticillioides* and *F. proliferatum*, few strains of *F. fujikuroi* and *F. andiyazi* were only able to synthesize trace amounts of these mycotoxins. Gibberellin A3 was, on the other hand, only produced by *F. fujikuroi* strains. These findings provide additional information on the variation within the *G. fujikuroi* species complex associated with rice with respect to ecology, phylogenetics, pathogenicity and toxigenicity.

INTRODUCTION

Rice (*Oryzae sativa* L.) is one of the most important crops in the human diet. Almost half the world's population subsists on rice as the major source of food. Although Asia accounts for most of the production and consumption (Faostat, 2008), rice is also a crucial staple and a source of income for millions of poor people in Africa and South America.

Gibberella fujikuroi (Sawada) Ito in Ito & K. Kimura species complex are teleomorphs of *Fusarium* species from section *Liseola* (Nirenberg and O'Donnell, 1998; Leslie *et al*., 2006). No less than ten sexual fertile biological species (mating type populations A-J) belonging to *Fusarium* genus have been grouped within the *Gibberella fujikuroi* species complex (Leslie *et al*., 2007). As a group, species of the *Gibberella fujikuroi* species complex contain a number of plant pathogens that are distributed worldwide and attack many economically important agricultural and horticultural crops (Leslie, 1995).

Taxonomically, *G. fujikuroi* species complex has traditionally been separated using morphological and biological species differentiation concepts. Morphologically, *G. fujikuroi* species are separated based on e.g. conidium size and shape as well as structure of the conidiophores while biological differentiation is done based on the sexual cross-fertility (mating types) (Leslie *et al*., 2006). However, species identification, based on morphological and biological characters, has proved difficult (Leslie *et al*., 2006 and 2007) and time-consuming; extensive training and expertise are also required.

Phylogenetic characters, based on DNA sequencing of e.g. translation elongation factor 1-alpha (TEF1-alpha) (O'Donnell *et al*., 1998; O'Donnell *et al*., 2000), internally transcribed spacer regions in the ribosomal repeat region (Waalwijk *et al*., 1996), beta-tubulin (O'Donnell *et al*., 1998; O'Donnell *et al*., 2000), histone (Steenkamp *et al*., 1999), calmodulin (O'Donnell *et al*., 1998; O'Donnell *et al*., 2000) and gibberellin (Malonek *et al*., 2005) gene have increasingly being

applied for species differentiation. Additionally, anonymous DNA markers (Amoah *et al*., 1995; Kini *et al*., 2002; Carter *et al*., 2008) and differences found in the ability to produce extrolites (secondary metabolites) have also been used for distinguishing species in this species complex (Leslie, 1995; Moretti *et al*., 1996; Frisvad *et al*., 2008).

In rice, some *Fusarium* sp. of the *G. fujikuroi* species complex have been associated with the Bakanae disease (Amoah *et al*., 1995; Desjardins *et al*., 2000). The typical symptoms of Bakanae in rice seedlings are slender, chlorotic and elongated primary leaves which seem to be induced by the production of gibberellin by the pathogen (Ou, 1987; Amoah *et al*., 1995). However, not all infected seedlings show these symptoms, as crown rot is also seen in many seedlings, resulting in stunted rice plants (Ou, 1987; Amoah *et al*., 1995). Independent of the symptoms, most of the infected plants usually die without reaching maturity. If plants survive until maturity, they may bear empty panicles. Crop losses caused by Bakanae may reach up to 40% (Ou, 1987).

Three mating populations of *G. fujikuroi* species complex have been associated with Bakanae on rice. Mating type population A (anamorph, *Fusarium verticillioides* (Sacc.) Nirenberg) (Amoah *et al*., 1995; Desjardins *et al*., 2000), mating type population C (anamorph, *F. fujikuroi* Nirenberg) (Desjardins *et al*., 2000) and mating type population D (anamorph, *F. proliferatum* (Mats.) Nirenberg) (Amoah *et al*., 1995; Desjardins *et al*., 2000). Identification of *G. fujikuroi* species causing the disease have proved to be problematic, since difficulties in obtain fertile crosses in mating populations associated with infected rice have been reported (Desjardins *et al*., 1997); at least two of the species (*F. fujikuroi* and *F. proliferatum*) are almost morphologically impossible to differentiate and are closely related regarding gene sequences (Leslie *et al*., 2006). Furthermore, it is not yet clear if all the three *Fusarium* species are associated with the symptoms of Bakanae of if *F. verticillioides* and *F. proliferatum* are present only as saprophytes.

Currently, there is limited information on occurrence and population structure of *G. fujikuroi* species complex associated with rice seeds originating from Africa and Asia. Some data

are available on the characterisation of *G. fujikuroi* species complex populations associated with rice seeds from Nepal (Desjardins *et al*., 2000), few strains were reported associated with rice seeds from Burkina Faso (Kini *et al*., 2002) and with rice seedlings from Ghana (Amoah *et al*., 1995).

In addition to affecting crop yield, strains of the *G. fujikuroi* species complex are also able to produce a number of mycotoxins (including fumonisins) (Gelderblom *et al*., 1988; Desjardins *et al*., 1997; Baird *et al*., 2008) and other bioactive secondary metabolites, such as gibberellin A3 (gibberellic acid) (Desjardins *et al*., 2000; Malonek *et al*., 2005). Fumonisins are known to be harmful for human and animal health due to their carcinogenic properties (Gelderblom *et al*., 1988) and presently they are among the most important toxins regarding food safety (EU, 2007). In rice, mycotoxin contamination has been associated with a high moisture content and poorly dried and stored grain (Desjardins *et al*., 2000); especially unpolished rice grains are more prone to fumonisin contamination (Abbas *et al*., 1998).

The present study had five objectives: i) to determine incidence and severity of *G. fujikuroi* populations associated with rice seeds from Africa and Asia; ii) to assess species composition and genetic variability of the strains using translation elongation factor 1-alpha (TEF) DNA sequences; iii) to evaluate pathogenicity of the different *Fusarium* species and strains on rice iv) to determine the ability of the strains to produce fumonisins and gibberellin A3 *in vitro* and v) to correlate the obtained molecular and mycotoxin profiles with the pathogenicity of *G. fujikuroi* species on rice.

RESULTS

Identification and incidence of *G. fujikuroi* species complex in rice seed samples

Strains of *G. fujikuroi* species complex were isolated from rice seed samples from Asia (China, Vietnam, India and Nepal) and Africa (Tanzania, Ghana, Burkina Faso and the Ivory Coast) (Table 1). The strains were characterised morphologically based on the production of whitish mycelium

growth, the presence of microconidia in chains (on monophialides or on polyphialides) and orange to salmon pionnotes on the seeds. The frequency of isolation of *G. fujikuroi* species complex in rice seeds varied according to the origin of the samples and was found to be 15% (Bangladesh), 40% (Ghana, India and Nepal), 60% (Burkina Faso and Tanzania), 67% (China and Vietnam) and 83% (Ivory Coast) (Table 2). The infection rate range was generally low and varied from 0.25% to 9% (Table 2). More specifically, the infection rates ranged from 0.5-7.5% in Bangladesh, 0.25-2% in Burkina Faso, 0.5-3.5% in China, 0.5-4.5% in Ghana, 0.5-1% in India, 0.5-6.5% in the Ivory Coast, 1.5-3% in Nepal, 1-2.25% in Tanzania and 0.5-9% in Vietnam (Table 2).

Phylogenetic analysis using DNA sequencing of the translation elongation factor 1-alpha (TEF) gene

All strains yielded an approximately 650bp fragment after PCR amplification with the translation elongation factor 1-alpha (TEF) gene primer pair. Phylogenetic analysis using Mega 4.0 generated one most-parsimonious tree from 592bp of aligned DNA sequences (Figure 1). This tree consisted of 4 different clades (Figure 1). Clade I comprised of *Fusarium verticillioides* strains originated from Africa and Asia. *F. verticillioides* was the most commonly found *Fusarium* species (13 out of 35 isolated strains) associated with rice seeds. The bootstrap value for this clade indicated 99% unity. Clade II, with bootstrap value of 99%, consisted of *F. andiyazi* Marasas, Rheeder, Lampr., K.A. Zeller & J.F. Leslie (5 strains out of 35 samples examined). Clade III was formed by *F. proliferatum* strains with 93% bootstrap support. *F. proliferatum* was the third most commonly species associated with rice seeds, found in 7 out of 35 isolated strains. Clade IV was composed by *F. fujikuroi* strains, originating from Asian countries only, with bootstrap value indicating 53% unity. *F. fujikuroi* was the second most common species associated with rice seeds with 10 identified strains out of the 35 isolated.

Analysis of fumonisins and gibberellin A3

The results of fumonisins and gibberellin production by *G. fujikuroi* complex species isolated from rice seed samples are shown in Table 5 and Figures 2. The production of FB_1 was higher compared to FB_2 (Table 4). FB1 was produced at significant levels by almost all the strains of *F. verticillioides* (clade I) and *F. proliferatum* (clade II) on RC (Table 4). *F. fujikuroi* (clade III) and *Fusarium andiyazi*. (clade IV) only produced traces of fumonisins on RC (Table 4). Similar results were obtained in PDA and YES (data not shown). Production of gibberellin A3 was only detected in culture extracts of strains of *F. fujikuroi* (clade III) (Figure 2). For most of the strains, RC and PDA seem to be the more suitable for gibberellin A3 production compared to YES (Figure 2).

Pathogenicity tests

All strains inhibited seed germination compared to the control treatment which was set to 100% germination. The number of germinated seeds treated with the different strains ranged from 27-80% (Table 3). Strains grouping in the clade III (*F. proliferatum* strains) showed stronger ability to inhibit seed germination compared to the other three clades (Tables 3 and 4). The average number of germinated seed was 64.2%, 70.6%, 49.6% and 64% for clades I, II, III and IV, respectively (Table 4). Independent of the *Fusarium* species, all the selected strains were able to induce leaf chlorosis and or the presence of slender leaves (Table 3). However, the most pathogenic strains considering induction of these symptoms were found in clade IV (*F. fujikuroi* strains), which showed in average 72.6% of plants with chlorotic and or slender leaves (Tables 3 and 4). Strains found in clades I (*F. verticillioides*), II (*F. andiyazi*) and III (*F. proliferatum*) showed lower ability to induce these symptoms with averages of 30.3%, 32.1% and 33.9%, respectively (Table 4). While strains found in clades I, III and IV showed variable ability to produce crown and stem rot symptoms (Table 3 and 4), strains found in clade II (*F. andiyazi*) showed the lowest ability to induce these symptoms with significantly lower pathogenicity scores compared to the other 3 clades

(Table 3 and 4). All strains found to be pathogenic in rice were re-isolated as endophytes from rice seedlings 30 days after inoculation (data not shown).

DISCUSSION

The present data confirm the importance of *Gibberella fujikuroi* species complex associated with rice seed samples in Asia and Africa. Fungal contamination of rice seed samples with this group of pathogens remains a clear problem for farmers in developing countries. With the exception of seeds originating from Bangladesh, the incidence of *G. fujikuroi* in seed samples was high, ranging from 40% to 83%. Indeed, Bakanae has proved to be primarily seed-borne and high levels of seed infection have been previously found in rice seed samples (Desjardins *et al*., 2000). However, in the present study, the infection rate range was low and varied from 0.2 to 9%. According to Mathur and Manandhar (2003), *F. moniliforme* as defined by Snyder and Hansen (which today consists of species within the *G. fujikuroi* species complex) infects rice seed samples at low ranges and are among the most common pathogen of rice worldwide.

DNA sequencing of the translation elongation factor 1-alpha (TEF) gene proved to be an efficient method for identification of *Gibberella fujikuroi* species complex associated with rice seed. It showed that the population diversity of *G. fujikuroi* species complex associated with rice within a country (e.g. China and Vietnam) was generally at least as diverse as that found across the countries. With the exception of strains isolated from Ghana and Bangladesh, strains obtained from rice seed of the same origin grouped differently, independently of the geographic source. All three species of *Fusarium*, namely *F. verticillioides* (*G. fujikuroi* mating type population A), *F. fujikuroi* (*G. fujikuroi* mating type population C) and *F. proliferatum* (*G. fujikuroi* mating type population D), commonly found associated with Bakanae disease of rice (Amoah *et al*., 1995; Desjardins *et al*., 2000), were found associated with the different rice varieties. *F. verticillioides* was the most frequently found species, followed by *F. fujikuroi* and *F. proliferatum*, suggesting that these species may play a significant role in the complex symptoms of Bakanae disease of rice. Additionally, *F.*

193 *andiyazi*, originally identified in sorghum in Africa (Marasas *et al*., 2001), was also isolated from
194 rice seed samples in the present study. According to Marasas *et al*. (2001), this species is closely
195 related to *F. thapsinum* Klittich, J.F. Leslie, P.E. Nelson & Marasas, which is found within the
196 *Gibberella fujikuroi* species complex. With the exception of *F. fujikuroi* strains, which was only
197 detected in seed samples originating from Asia, all the other *Fusarium* species identified were
198 found in both African and Asian seed samples.

199 In the present study, four different genetic clades were identified (Figure 1). While
200 clade I only contained *F. verticillioides* strains, clade II grouped no more than *F. andiyazi* strains.
201 The high bootstrap values obtained for these two clades suggested high genetic similarity of the
202 strains within each clade (Figure 1). Similar information is valid for the clade III that consists of
203 other *F. proliferatum* strains. On the other hand, clade IV that contained *F. fujikuroi* strains was the
204 most diverse genetically with bootstrap value of 53%.

205 The observed production of fumonisins *in vitro* by our strains is consistent with
206 observations of Desjardins *et al*. (1997) who reported significant fumonisin production by *F.*
207 *proliferatum* and *F. verticillioides* but very limited by *F. fujikuroi*. Production of fumonisins (FB1,
208 FB2 and FB3) by *F. proliferatum* strains isolated from rice has also been reported by Abbas *et al*.
209 (1998, 1999). Also, coincidently with our results, Park *et al*. (2005) reported the production of
210 fumonisin FB1 by strains of *F. verticillioides* and *F. proliferatum* isolated from rice. In addition to
211 FB1 and FB2, several additional fumonisin analogues were detected, including FB3 and FA1 (data
212 not shown). The production of these analogues was linked to production of FB1 and FB2, with the
213 predominant FB1 and FB2 producers also being the best producers of FB3 and FA1. Although
214 strains of *F. fujikuroi* and *F. andiyazi* were weak producers of fumonisins, they were still
215 pathogenic to rice (Table 3), suggesting that other compounds may play a more significant role in
216 pathogenicity for the low-producing strains. In addition to fumonisins and gibberellin A3, the
217 metabolic profiles of the strains isolated from rice showed that they can produce many other

metabolites including moniliformin, fusaric acid, fusarin A and C, fusaproliferin and beauvericin (data not shown).

In the present study, only strains of *F. fujikuroi* grouped in clade IV were able to produce gibberellin A3. These results are in agreement with previous reports about gibberellin production of *G. fujikuroi* species complex in rice (Desjardins *et al*., 2000; Malonek *et al*., 2005). According to Malonek *et al*. (2005), although most of the anamorphs within *G. fujikuroi* species complex contain the entire genome for synthesis of gibberellins, only *F. fujikuroi* and single strains of *F. konzum* Zeller, Summerell & J.F. Leslie can produce this metabolite. Rim *et al*. (2005) also reported that a single strain of *F. proliferatum* (identified using the internal transcribed spacer region of the ribosomal gene) as a gibberellin producer. The production of gibberellin by *F. fujikuroi* strains has been associated with the disease symptom of abnormal elongation of rice plants (Ou, 1987). In our study, strains of *F. fujikuroi* showed higher percentages of plants with chlorotic and slender leaves compared to the other strains (Table 3), supporting the idea that gibberellin may contribute to the expression of these symptoms. Furthermore, *F. fujikuroi* was, in the present and other studies (O'Donnell *et al*., 1998), found to be phylogenetically closely related to *F. proliferatum*, but the distinct ability to produce secondary metabolites, such as fumonisins and gibberellin A3, suggests that these two species may be biologically different.

The data obtained from the pathogenicity tests showed that all 35 selected strains belonging to the *G. fujikuroi* species complex affected seed germination in rice. However, strains of *F. proliferatum* (clade III) showed significantly higher ability to inhibit seed germination in rice compared to the other *Fusarium* species. Furthermore, populations of *G. fujikuroi* species complex caused symptoms of chlorotic and/or slender leaves with and without induction of crown and stem rot, indicating varying ability of the strains to cause symptoms in rice. According to Amoah *et al*. (1995), the type of symptoms caused by *G. fujikuroi* species complex in rice (and maize) may depend on the balance of toxins and growth regulators which are, on the other hand, affected by the

strain of the pathogen and the nutritional status of the plant and the environment. Considering the ability to induce Bakanae symptoms (chlorotic and slender leaves as well as crown/stem rot symptoms), *F. fujikuroi* (clade IV) was the most pathogenic species to rice compared to *F. verticillioides* (clade I), *F. andiyazi* (clade II) and *F. proliferatum* (clade III). However, all the four presently identified Fusaria should be considered as potential pathogens of rice and part of this complex disease, as some individual strains of *F. verticillioides*, *F. andiyazi* and *F. proliferatum* showed as aggressive as *F. fujikuroi* in rice (Table 3). Furthermore, all pathogenic strains were able to infect rice seedlings from the inoculated seeds and could be found systemically, growing endophytically within the plant.

The present information provides further knowledge on the ecology, phylogenetics, pathogenicity and toxigenicity of *G. fujikuroi* species complex in rice. A more detailed analysis of the obtained gene sequences may facilitate the development of more accurate tools to diagnose the disease, contributing thus to the improvement of strategies to manage Bakanae. The current work also clearly showed that some strains of *F. verticillioides*, *F. andiyazi* and *F. proliferatum* are not merely saprophytes as they can cause symptoms similar to the ones caused by *F. fujikuroi*. As rice is a staple crop for many people worldwide and some of the presently isolated strains are able to produce fumonisins, the importance of this mycotoxin in rice needs to be clarified. A more detailed examination of the ability of *G. fujikuroi* species complex to produce fumonisins as well as other mycotoxins *in planta* warrants further investigation.

EXPERIMENTAL PROCEDURES

Seed samples and fungal isolation

Seventy-four rice (*Oryzae sativa* L.) seed samples originating from smallholder farms in different countries in Asia and Africa were examined for the presence of anamorphs of the *Gibberella fujikuroi* species complex. Four-hundred seeds were plated on sterile water soaked blotters (filter

papers) in Petri dishes and incubated at room temperature (22^{o}C) for 7 days under near ultra violet (NUV) light (Mathur and Kongsdal, 2003). Seeds infected with *G. fujikuroi* were detected using stereo and compound binocular microscopes, and one strain per seed was isolated on potato dextrose agar (PDA) supplemented with streptomycin sulphate (0.3g of streptomycin sulphate in 1000ml PDA) (Samson *et al*., 2002). Preliminary identification of the strains was based on habit characters and on morphological characters of the fruiting bodies and spores/conidia (Mathur and Kongsdal, 2003). Fungal strains were collected from 42 rice seed samples of which 35 strains were selected (Table 1) for further studies due to pathogenicity on rice. The selected strains were sub-cultured once again on PDA before production of single spores.

From the Blotter tests, the frequency of isolation (%) and infection rate range (%) were quantified. The frequency of isolation (%) was calculated based on the total number of samples tested per country and number of these samples found infected with *G. fujikuroi* species complex. The infection rate range (%) was determined by assessing the number of seeds found infected with *G. fujikuroi* species complex in a sample of 400 seeds.

Single spore cultures

Two week-old fungal culture, growing on PDA and under NUV light, was flooded with 10ml sterile water and gently scraped with a flamed Drigalski spatula. The obtained inoculum suspension was filtered through a three-layer sterilised gaze and 0.5ml of the suspension was used for serial dilution (10^{0} to 10^{-5}). One hundred µl of each dilution was plated on Special Low Nutrient Agar (SNA, Scharlau Microbiology, Spain). The plates were then incubated with the lid up at room temperature. Single spores with one to three germ hyphae (germling) were identified by a daily examination of the plates under a stereo microscope. Individual germlings were excised from the SNA plates with a sterile needle and transferred to a new PDA plate. The plates were then incubated under NUV light

293 at room temperature for one week. Suspension of single spore cultures were prepared with sterile
294 water and kept at -80^{o}C until use.
295
296 **Extraction of total genomic DNA**
297 Fifty ml of sterile PDB were inoculated with 500µl of the single spore culture suspensions.
298 Inoculated PDB flasks were incubated under agitation (100rpm) at room temperature for 3 days.
299 The fungal cultures were then filtrated in a polyethylene membrane and washed twice with sterile,
300 distilled water. The membrane with the mycelium was folded and quickly dried with a tissue paper.
301 The near dried fungal mycelium was transferred to an Eppendorf tube, frozen for 2h at -20^{o}C and
302 lyophilized over night. The lyophilized mycelium was then ground into a fine powder in liquid
303 nitrogen using sterile pestle and mortar. DNA was extracted from each sample using the Dneasy
304 Plant Mini Kit (Qiagen Group). The quality of the DNA was checked in agarose gels (1.7%) and the
305 quantity determined in a spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware,
306 USA).
307
308 **Species identification and phylogenetic analysis using translation elongation factor 1-alpha**
309 **(TEF) DNA sequences**
310 TEF-1 (forward primer: 5'-ATGGGTAAGGARGACAAGAC-3') and TEF-2 (reverse primer: 5'-
311 GGARGTACCAGTSATCATGTT-3') (O'Donnell *et al*., 1998) primers were used in the PCR
312 amplification assays. The PCR reaction (1x) consisted of 1µl DNA (10-100 ng DNA), 1µl of TEF-
313 1 (10 pmol/µl), 1µl of TEF-2 (10 pmol/µl), 0.3µl of Pfu DNA Polymerase (2.5U/µl, Fermentas,
314 EU) and 18µl of buffer mix [880µl of sterile MilliQ water, 100µl of 10x reaction buffer (200mM of
315 Tris-HCL pH 8.8, 100mM of $(NH4)_2SO_4$, 100Mm of KCl, 1% of Triton X-100, 1mg/ml of BSA,
316 20mM of $MgSO_4$) and 20µl of DNTP (10mM)]. The PCR programme had an initial DNA
317 denaturation step of 3 minutes at 94^{o}C, followed by 34 cycles of 30 seconds at 94^{o}C, primer
318 annealing for 45 seconds at 61^{o}C, primer extension for 1 minute at 72^{o}C. The last step for the final

primer extension reaction consisted of an incubation at 72°C for 15 minutes. The amplified DNA product was mixed with 2µl of loading dye 10x (0.25% of bromophenol, 0.25% of xylene cyanol and 40% of sucrose w/v) and separated by electrophoresis by running 2.5-5µl of the product on 1.7% agarose gel (Gibco, BRL) in 0.7 x TBE buffer (Sambrok *et al*., 1989) at 300V for approximately 45 min. The gels were stained in an ethidium bromide solution (1µg of ethidium bromide per ml 0.7 x TBE) and photographed under UV light for the visualisation of a 700bp band.

After amplification, PCR products were purified using the DNA Extraction Kit (Fermentas Life Science, Germany). The purified products were sequenced in both directions with TEF primers at Eurofins MWG Operon (Germany).

The obtained sequences were initially aligned in the Fusarium ID database (Geiser *et al*., 2004) available at http://isolate.fusariumdb.org/index.php to provide species identification. In the case of the identification of *Fusarium andiyazi* strains, as the Fusarium ID database was unable to identify these strains at species level (first ID option for all the 5 strains was *Fusarium* spp.), the programme Blast available at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used. Phylogenetic analyses were conducted using the software *MEGA* version 4 (Tamura *et al*., 2007). Confidence in the branching points was obtained by conducting 1,000 bootstrap replicates using maximum-parsimony as the criterion.

Production and analysis of secondary metabolites

Methanol, dichloromethane, ethyl acetate and acetonitrile (MeCN) were gradient grade and purchased from Sigma-Aldrich (St. Louis, MO). Water was purified with a Milli-Q system (Millipore, Bedford, MA). Formic acid was purchased from Sigma-Aldrich and was of 98% purity. A reference standard of a fumonisin mixture containing FB1 and FB2 was purchased from Biopure (Tulln, Austria), FB3 was a kind gift from Dr. Michael Sulyok, Center for Analytical Chemistry (IFA-Tulln, Austria) and Gibberellin A3 was obtained from (Amdal Company, Chicago, IL).

Thirty-five strains of the *Gibberella fujikuroi* complex isolated from rice were grown on Potato dextrose agar (PDA) at 25°C in darkness and after 7 days transferred by three-point inoculation to Yeast Extract Sucrose agar (YES) (Samson *et al.* 2002), Rice Corn Steep agar (RC) (Bullerman, 1974) and PDA. The strains were incubated for 14 days at 25°C in darkness. The metabolites of the strains were extracted using a modified version of the micro-scale extraction procedure for agar plugs previously described (Smedsgaard, 1997). Six plugs from each plate taken from the center of the colonies were transferred to 2ml HPLC vials and extracted with 1ml methanol:dichloromethane:ethyl acetate (1:2:3) containing 1% (v/v) formic acid. The plugs were extracted at -20°C overnight and ultrasonically for 1 hour at room temperature. The extracts were transferred to new vials and evaporated *in vacuo*. The plugs were re-extracted ultrasonically for 1 hour with 0.8 ml methanol:water (80:20). These extracts were used to re-dissolve the first extracts.

Liquid chromatography was performed on an Agilent HP 1100 HPLC system controlled by MassLynx V 4.0 (Waters-Micromass, Manchester, U.K.). Extracts of 2µL were injected and separated on a 50 × 2mm i.d., 3µm, Luna C18 II column (Phenomenex, Torrance, CA) using a MeCN and water gradient with a flow rate of 0.3 ml/min starting at 15% of MeCN, which was increased lineally to 100% of MeCN in 20 minutes, keeping this for 5 minutes before returning to start conditions in 3 minutes and equilibrating for 2 minutes. The solvents were buffered with 20mM of formic acid. The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe operating in the positive ESI mode (Nielsen and Smedsgaard, 2003). MS spectra were collected as centroid data from *m/z* 100 to 900, with a scan time of 1 s and an inter-scan time of 0.1 s. The protonated ions $[M+H]^+$ with an interval of mass ±0.02 amu were used for identification of all fumonisins, whereas $[M+H-H_2O]^+$ was used for gibberellin A3 as previously described (Nielsen and Smedsgaard, 2003). Other data were matched against an internal database of approx 750 reference standards as well as the 34 500 structures in Antibase2008 (John Wiley and Sons, inc).

Pathogenicity tests

Pathogenicity tests were conducted in seed inoculation assays. In total, three different experiments were conducted. Seeds of rice, cultivar Q5, were tested for the presence of seed-borne pathogens using the blotter method (Mathur and Kongsdal, 2003). Seed lots found free of seed-borne pathogens were used in the experiments (data not shown). Seeds were surface disinfected by immersion in 70% ethanol for 1 minute, transferred to 1% sodium hypochlorite for 3 minutes and rinsed three times consecutively in sterile distilled water. Seeds were then left to dry in the flow cabinet in Petri dishes containing sterile filter paper. Fungal inoculum suspension was prepared for each strain from the single spore cultures. The concentration of the inoculum suspension was determined using a hemacytometer and adjusted to 1 X 10^5 spores/ml with sterile water.

Twenty rice seeds were soaked in 10ml of inoculum suspension for 18 hours at room temperature. Control seed were soaked in sterile water. Inoculated and control seeds were sown into 100ml plastic pots (4 pots per isolate/ 4 seeds per pot). Each pot, containing 75g autoclaved mixture of soil and sand (3:1), was regularly watered with a suspension containing liquid fertiliser (20ml liquid fertiliser per 1L water) (Hornum Næring, Brøste A/S, Denmark). The trays were incubated in a humid chamber at 27 ^{o}C. Seed pots were kept in trays covered with plastic for one week, thereafter the plastic was removed. Fifteen days after inoculation, the number of germinated seeds was assessed. The seedlings were observed for symptoms of Bakanae (number of slender and chlorotic leaves and number of plants showing crown rot) 15 and 30 days after inoculation.

In order to test for the Koch's postulate, symptomatic seedlings were excised (4 plants per treatment) just above the soil line, 30 days after inoculation. The stem part was cut in approx. 1cm segments which were surface disinfected for 1min in NaOCl and washed 3 times in sterile, distilled water. The stem parts were then transferred to a plastic bag, containing 1ml of sterile water and mortared with a pestle. One-hundred µl of the stem extract was plated on PDB supplemented

with streptomycim (4 plates per treatment). Plates were then incubated at room temperature and 3-5 days later, they were assessed for the presence of fungal colonies.

Analysis of variance in the pathogenicity tests was conducted using the General Linear Models (GLM) of the Statistical Analysis Systems (SAS) package (Statistical Analysis Systems Institute Inc., Cary, NC, USA) using 16 repetitions per treatment. The different clades and treatments were examined for statistical significance using the Student-Newman-Keuls and the Least Square Means test at $P \leq 0.05$.

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496 Table 1. Fungal strain number, DSHC accession number, IBT collection number, rice variety
497 and local origin of *Gibberella fujikuroi* species complex associated with rice seeds.

498

Strain number	**DSHC accession number**	**IBT collection**	**Rice variety**	**Origin**
1ALH	47.795	41367	FKR 18	Burkina Faso
7ALH	47.690	41368	Khumal 11	Nepal
20ALH	44.947	41370	TNDB 100	Vietnam
21ALH	44.494	41371	CM 1490	Vietnam
22ALH	47.532	41372	TOX 3393-64-2-21	Ivory Coast
23ALH	47.542	41373	WAB	Ivory Coast
24ALH	47.552	41374	Bouake 189	Ivory Coast
25ALH	47.557	41375	Sresre	Ivory Coast
26ALH	47.701	41335	Khumal 4	Nepal
28ALH	48.021	41337	Sunjin	China
29ALH	48.487	41338	Feng Yuan You 272	China
30ALH	48.483	41339	Long Dao	China
31ALH	48.468	41340	Sang You 361	China
32ALH	48.469	41341	Sang You 22	China
33ALH	48.475	41342	II You 838	China
34ALH	48.479	41343	Yi Xiang 2292	China
35ALH	48.480	41344	Yi Xiang 99E-4	China
36ALH	48.472	41345	II You 6078	China
37ALH	48.482	41346	Hong You 131	China
38ALH	48.445	41347	CTH-1	India

39ALH	48.444	41348	KH-50	India
41ALH	48.493	41350	Supa	Tanzania
44ALH	48.507	41353	Saro	Tanzania
45ALH	48.508	41354	Supa	Tanzania
46ALH	48.249	41355	No. R12	Ghana
47ALH	48.241	41356	No. R5	Ghana
49ALH	48.525	41357	D uu 527	Vietnam
51ALH	48.538	41359	Nam Dinh 1	Vietnam
52ALH	48.541	41360	DT 10	Vietnam
53ALH	48.543	41362	Q uu 1	Vietnam
53.2ALH	48.548	41361	IR 35366	Vietnam
54ALH	48.552	41363	OM 2517	Vietnam
55ALH	48.573	41364	BR 5	Bangladesh
56ALH	48.574	41365	BR 5	Bangladesh
57ALH	48.586	41366	327-Ri-07	Burkina Faso

502 Table 2. Frequency of isolation (%) and infection rate range (%) of *Gibberella fujikuroi* species
503 complex strains isolated from rice seeds originating from Asia and Africa
504

Origin	Number of samples tested	Number of samples found infected	Frequency of isolation (%)	Infection rate range[1)] (%)
Bangladesh	13	2	15	0.50-7.50
Burkina Faso	5	3	60	0.25-2.00
China	15	10	67	0.50-3.50
Ghana	5	2	40	0.50-4.50
India	5	2	40	0.50-1.00
Ivory Coast	6	5	83	0.50-6.50
Nepal	5	2	40	1.50-3.00
Tanzania	5	3	60	1.00-2.25
Vietnam	15	10	67	0.50-9.00

505
506 [1)] Percentage of seed per sample found infected with species of the *Gibberella fujikuroi* species complex

507 Table 3. Pathogenicity effect of *Gibberella fujikuroi* species complex strains in rice seedlings

STRAIN NUMBER	IDENTIFICATION [1)]	GENETIC CLADE	NUMBER OF GERMINATED SEEDS (%)	NUMBER OF CHLOROTIC AND SLENDER LEAVES (%)	CROWN AND STEM ROT INCIDENCE (%)
53.2ALH	*F. verticillioides*	**I**	80	17.0	0.0
52ALH	*F. verticillioides*		73	37.5	10.0
35ALH	*F. verticillioides*		47	29.0	14.5
56ALH	*F. verticillioides*		40	35.0	46.5
22ALH	*F. verticillioides*		67	44.5	53.0
47ALH	*F. verticillioides*		80	32.5	18.0
31ALH	*F. verticillioides*		67	35.0	3.0
36ALH	*F. verticillioides*		73	22.5	0.0
39ALH	*F. verticillioides*		27	50.0	3.0
25ALH	*F. verticillioides*		87	32.0	29.0
32ALH	*F. verticillioides*		67	10.0	25.0
23ALH	*F. verticillioides*		80	13.5	13.0
46ALH	*F. verticillioides*		47	36.0	35.5
30ALH	*F. andiyazi*	**II**	53	40.5	0.0
53ALH	*F. andiyazi*		80	23.5	0.0
38ALH	*F. andiyazi*		73	22.5	3.0
44ALH	*F. andiyazi*		67	45.0	5.0
1ALH	*F. andiyazi*		80	29.0	5.5
51ALH	*F. proliferatum*	**III**	53	33.5	24.5
45ALH	*F. proliferatum*		67	60.0	39.9
24ALH	*F. proliferatum*		47	27.0	34.5
41ALH	*F. proliferatum*		60	25.5	17.5
34ALH	*F. proliferatum*		47	7.0	0.0
54ALH	*F. proliferatum*		33	46.5	62.5
57ALH	*F. proliferatum*		40	37.5	65.5
7ALH	*F. fujikuroi*	**IV**	60	58.5	62.5
37ALH	*F. fujikuroi*		80	74.0	16.5
20ALH	*F. fujikuroi*		53	84.0	52.5
29ALH	*F. fujikuroi*		60	60.5	13.0
55ALH	*F. fujikuroi*		60	69.5	69.0
26ALH	*F. fujikuroi*		67	72.5	59.5
21ALH	*F. fujikuroi*		53	46.5	23.0
49ALH	*F. fujikuroi*		67	95.0	20.0
33ALH	*F. fujikuroi*		73	89.5	9.5
28ALH	*F. fujikuroi*		67	76.5	35.5

508
509 [1)] Identification based on gene sequencing of the translation elongation factor 1-alpha (TEF) gene.

510

511 Table 4. Pathogenicity effect of *Gibberella fujikuroi* species complex in rice seedlings grouped in 4

512 different genetic clades

GENETIC CLADE	MEAN OF GERMINATION (%)	MEAN OF NUMBER OF CHLOROTIC AND SLENDER LEAVES (%)	MEAN OF CROWN AND STEM ROT INCIDENCE (%)
I	64.2 a	30.3 b	19.3 ab
II	70.6 a	32.1 b	2.7 b
III	49.6 b	33.9 b	34.9 a
IV	64.0 a	72.6 a	36.1 a

513

514 Means were statistically compared using the Student-Newman-Keuls test ($P \leq 0.05$). Values followed by the same letters

515 were not significantly different from each other

516 **Table 5**. Production of fumonisin B1 and B2 by *G. fujikuroi* complex from rice on Rice Corn Steep

517 Agar (RC)[a].

No	Species	FB1	FB2
7	*F. fujikuroi*	trace	trace
20	*F. fujikuroi*	trace	trace
21	*F. fujikuroi*	+	trace
26	*F. fujikuroi*	trace	trace
28	*F. fujikuroi*	+	trace
33	*F. fujikuroi*	trace	trace
37	*F. fujikuroi*	trace	trace
49	*F. fujikuroi*	trace	trace
55	*F. fujikuroi*	trace	trace
24	*F. proliferatum*	++++	+++
34	*F. proliferatum*	+++	++
41	*F. proliferatum*	++++	+++
51	*F. proliferatum*	++++	++++
54	*F. proliferatum*	++++	+++
57	*F. proliferatum*	trace	trace
1	*F. andiyazi*	trace	trace
38	*F. andiyazi*	trace	trace
44	*F. andiyazi*	++	trace
50	*F. andiyazi*	trace	trace
53	*F. andiyazi*	trace	trace
22	*F. verticillioides*	++	++
23	*F. verticillioides*	++++	++
25	*F. verticillioides*	++	+
31	*F. verticillioides*	+++	++
32	*F. verticillioides*	+++	++
35	*F. verticillioides*	++	++
36	*F. verticillioides*	++	+
46	*F. verticillioides*	+++	++
47	*F. verticillioides*	++++	++
52	*F. verticillioides*	+++	++
53,2	*F. verticillioides*	+++	++
56	*F. verticillioides*	+	trace

[a] Trace(<0.1 µg/ml), + (0.1-1 µg/ml), ++ (1-10 µg/ml), +++ (10-25 µg/ml) and ++++ (>25 µg/ml).

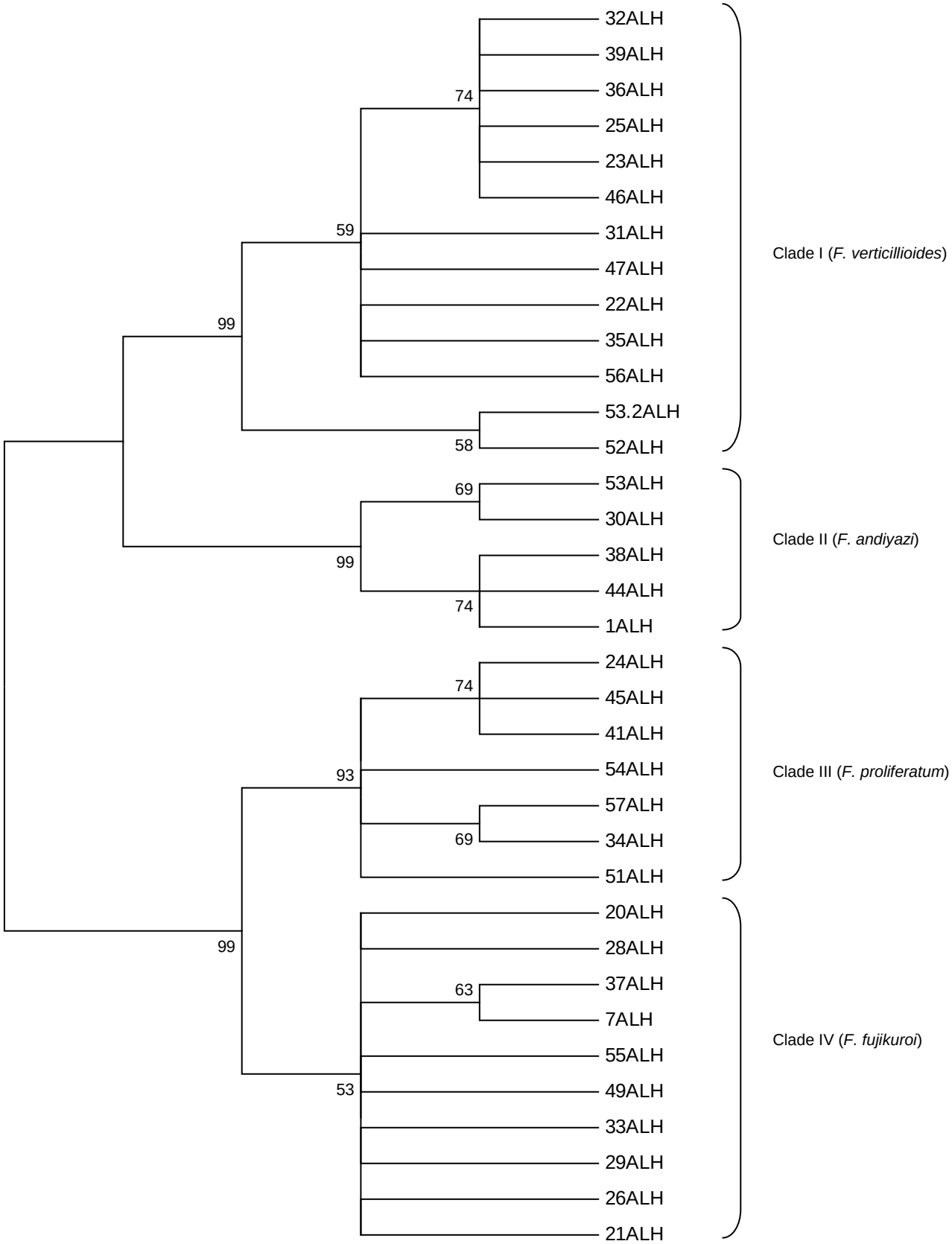
518

519

FIGURE LEGENDS

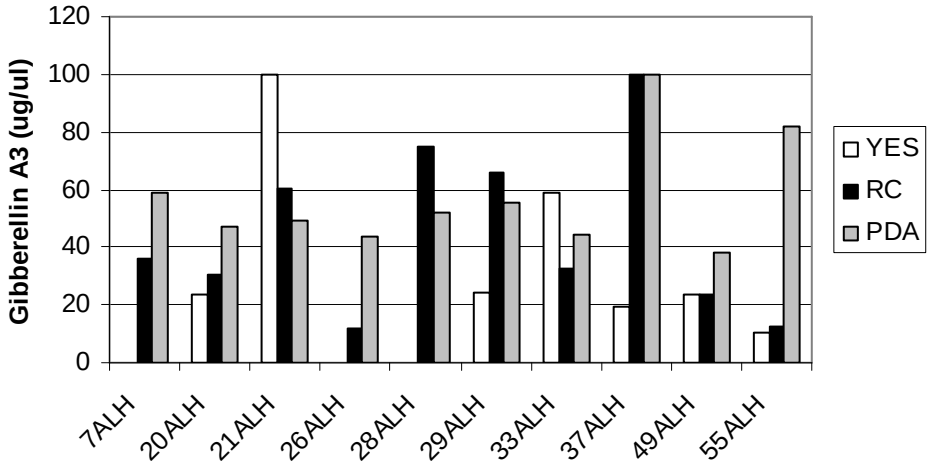
Figure 1. Maximum-parsimony tree based on single-gene sequences of the translation elongation factor 1-alpha gene from *G. fujikuroi* species complex associated with rice seeds. Bootstrap values based on 1000 replications are indicated as percentages in the internodes when replication frequencies exceed 50%

Figure 2. Production of gibberellin (A3) by *G. fujikuroi* species complex from rice in Yeast Extract Sucrose Agar (YES), Rice Corn Steep Agar (RC) and Potato Dextrose Agar (PDA) in (%)



531

532 Figure 1



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534 Figure 2